BASIC METHODS
FOR
EXPERIMENTS ON EGGS
OF
MARINE ANIMALS

JUST

A. R. Menhard

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By
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Preface

In this book I set forth fundamental methods for the use of eggs and spermatozoa of marine invertebrates in experimental investigation. In addition to methods concerning marine gametes generally, I give some that refer especially to those eggs and spermatozoa that are most commonly employed; these methods, however, are themselves so general that they apply equally to gametes of the same species both in American and in European waters, and to those of closely related species. The book thus gives methods for any species of marine gametes used in laboratory investigation.

The book in no wise aims to be one of recipes for individual experiments or a catalogue of all methods in use. Instead, it purposes to pass on to the reader what I have learned during my years of experience with marine eggs. The individual experiment capable of being repeated at will depends very strongly on the adherence to some general rules. These I here set forth. They will serve both as the starting-point for one who for the first time enters the field of experimental embryology, and as a basis for the specialist whose work demands individual extension of methods.

The suggestion to make my knowledge of methods for successful experiment on marine eggs more generally available came from a number of fellow-workers. The strongest impetus was received from my friends, Mr. Ware Cattell and Dr. L. V. Heilbrunn. In answer to their insistence I published some years ago a series of articles in Mr. Cattell's "*Collecting Net.*" In the years before these articles appeared I had had at Woods Hole much experience in aiding investigators who frequently sought my advice. In this wise I came to know more surely what are the chief needs of those who work on marine eggs. Since the publication of these notes many investigators both in America and in Europe have requested their re-issue in a more available form. In meeting this demand I have made additions to the

original notes from my experience gathered in the meantime at Woods Hole, Naples and Roscoff.

For many problems in embryogenesis, the study of the living egg alone does not suffice or is impossible to make. Study of the fixed egg thus becomes a necessary supplement or is mandatory. Methods for the preparation of the fixed egg are therefore indispensable for the experimental embryologist. Hence, a large section of this little book is devoted to these methods.

The methods detailed are all known to me through years of experience. Some of my own devising have given preparations whose superlative beauty has elicited most favorable comments from experts in cytology both in Europe and in America. I may point out that during more than twenty-five years I have used these methods with unvarying success on animal cells, from Protozoa to man included. They are therefore of value to cytologists generally.

Since the book is not encyclopedic in its scope, it carries no literature list. Some few references to original papers are added at the ends of sections. These references contain additional citations to literature.

I wish to express my best thanks to Dr. L. V. Heilbrunn who so kindly consented to correct the proof.

E. E. J.

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de Roscoff
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Contents

I

GENERAL WORKING CONDITIONS, PRECAUTIONS AND PREREQUISITES

The first rule to be observed by the experimental embryologist is that of scrupulous cleanliness. Contamination in all forms must be avoided. It represents a serious source of error. *Be at great pains to insure the absolute cleanliness of every utensil used.* This precaution is as important as any taken against accidental contamination during the actual experimental work.

Glassware

Ordinarily one uses the common cleaning fluid, potassium bichromate and sulphuric acid. Because of its great toxicity, the least trace remaining on the glassware is deleterious to living cells. Whenever it has been used, it must be thoroughly removed, preferably by a prolonged washing in running water with a final rinsing in distilled water. The same precaution must be taken with nitric acid. After its use, the glassware should be washed in running water until the most delicate test for acid gives no reaction. Soap and soap powders, if not thoroughly removed, give, when the glassware is used for sea-water, a precipitate which adhering to the vessel will modify the experimental results. I find that Bon Ami is superior to soaps and soap powders because more readily removed. It leaves the glass dishes bright and clear. My practice is to scrub the dishes vigorously with Bon Ami two or three times, rinsing after each scrubbing, then finally to apply a suspension of the Bon Ami to the glassware, setting it aside until thoroughly dried. Then the dried powder is removed with dried towels, after which the glassware is rinsed in running water for several minutes. *The glassware is then stacked upright, and never upside down for fear of chance contamination*, on dry linen towels

which have been previously rinsed in running water. Glassware may also be stacked on glass plates. This is the practice of many workers. Because of the alkali that they may still contain, *I never use laboratory towels for drying glassware without having previously washed them in running water.*

Before using dishes which are to hold eggs or sperm, one should rinse them first in running tap water and then in sea-water. *After finishing an experiment or observation, wash dishes used first thoroughly in sea-water and then in running tap water*, because cytolyzed eggs or sperm may stick to the glass, if it is not washed in sea-water first.

All glassware used should be of the best grade procurable. If new dishes are not available, one must take added precautions against possible contamination. It often happens that a previous worker has thoughtlessly used dishes as receptacles for toxic agents without cleansing them afterwards. Dishes for experimental work on living eggs should never be used as slop jars. Chemicals added to sea-water for an experiment should be thoroughly removed. However, there is quite a difference in the degree to which glass adsorbs various toxic substances. Thus I have reared *Platynereis* embryos to sexual maturity in dishes borrowed from another worker who had kept waste Bouin's fixing fluid in them for several weeks. After I had washed these dishes thoroughly, they were fit for my purpose. Corrosive sublimate solutions, on the other hand, are more difficult, if not impossible, to remove.

As far as possible one should use the same kind of glassware throughout a given series of experiments. It is of importance, for example, to have vessels of the same size and capacity. If, in a series, one were to use vessels all of the same capacity in cc. but of different heights, there would obviously be a difference in the distribution of the eggs in the vessels. Depending upon the diameter of the bottom of the vessels, the eggs will be closely crowded, perhaps in more than one layer, or widely scattered. Moreover, with the same volume of sea-water in each vessel, there will be a difference in depth of the water as well as a difference in the surface exposed. If, on the other hand, in the given series of an experiment one uses vessels of varying capacities, again, even though the same volume of eggs be

present thoroughout the series, differences enter which may be responsible in some measure for the results obtained.

I find it a good practice, therefore, to use dishes as nearly uniform as possible. For this reason one may select the Fostoria finger bowls having as nearly as possible the same kind of bottoms. Those, the bottoms of which are too concave or too convex, should be rejected. These finger bowls are used, as will be shown beyond, when certain volumes of eggs are studied. One may also use low stender dishes which come in varying capacities in cc. These have a certain advantage because they possess well-fitting covers.

The Syracuse watch-glass is extremely useful. Since it is so heavy and perfectly flat, it can not be upturned. Moreover, the eggs are never overcrowded, as they are, for instance, in the Boveri dishes. Nowadays one can also obtain laboratory ware in quartz. For some work quartz dishes, slides, capillary tubes and cover-slips are most valuable. For work on sperm, for example, I use quartz exclusively.

The ordinary pipettes used for handling eggs or sperm should be the best obtainable. The glass tube should be long enough so that when water is drawn up it never reaches the rubber bulb. It is a good plan to calibrate such pipettes in some units, as for example, at intervals of 0.5 cc. There should be one pipette for each dish in the experimental series, all of which are similarly calibrated. *For eggs use pipettes with large orifices; small bore pipettes are apt to injure the eggs. Do not ever force eggs through pipettes.* For spermatozoa it is preferable to use pipettes with small bore, especially where the quantity of spermatozoa is small.

The graduated cylinders of the same volume should be of the same height. This is especially desirable if one is collecting eggs, since the time to their complete settling obviously varies with the height of the cylinder. I use the 250 cc. cylinder most frequently.

The selection of a certain size and capacity of dishes depends upon the volume of eggs used in the experiment. If one works with volumes of eggs up to 1 cc., one should best use finger bowls which hold 250 cc. of sea-water without being completely filled. However, if one has to do with only a few hundred eggs

of the size of those of *Arbacia* or *Cumingia*, it is preferable to use stenders of 100 cc. capacity or less, depending upon the number of eggs.

In selecting the dishes one should also consider the species of egg under investigation. Some eggs develop normally or not depending upon the volume of sea-water containing them. Thus the eggs of *Asterias*, removed from the ovary, go through most normal maturation when placed in larger volumes of sea-water. (Just, 1929.) For the eggs from one ovary of *Asterias* I used flat-bottom dishes which easily hold 3,000 cc. of sea-water. On the other hand, eggs of *Platynereis megalops* will fertilize only when in a volume of sea-water equal to and not surpassing the volume of eggs. (Just, 1915.)

Utilization of Sea-Water

Precautions must be taken to insure as far as possible uniformity with respect to the temperature and gas content of the sea-water. Heilbrunn's practice of drawing off in a large flask the sea-water that he is later to use in an experiment is excellent. In the first place, this sea-water soon comes to room temperature or at least insures that all dishes in the series of a given experiment have sea-water of the same temperature. Secondly, sea-water is frequently charged with gas as it comes from the taps. Therefore, if we follow Heilbrunn's method, by the time that we are ready to use the sea-water, the gas bubbles have disappeared. When present they are a nuisance and may be even harmful. *Sea-water should never be drawn from the tap directly on to the egg suspension; the eggs may thus be injured.*

Dishes containing eggs should always be protected against evaporation, because this makes the sea-water hypertonic and hypertonic sea-water is itself an experimental method. (Just, 1928.) Therefore the dishes should be covered. On warm days it is well to keep such dishes on the live table in running sea-water which, however, should never be too deep. Where eggs are in cylinders, test tubes or small dishes, they may be placed in a larger dish with wetted sea-sand on to which a gentle stream of sea-water flows. The containers, of course, must be covered to avoid chance entrance of sea-water, and precautions must be taken against overflow. That is, the cylinders, tubes or other

vessels should be placed in the sand in such wise that the tops are well above the surface of the sand.

Dishes containing eggs should, of course, be protected against direct sunlight.

Thermometers

It is well to record both the room temperature and that of the sea-water containing the eggs. One should therefore have available two standardized thermometers. If possible, one should use such which read in tenths of degrees. The room temperature should be taken always in the same place in the room which should be protected against sunshine. The dishes containing the eggs, the temperature of which is taken, should be similarly placed and protected. In view of the fact that change in temperature is a very important experimental means, the worker should keep careful record of the temperature at which he conducts his experiments.

Optical Equipment

Many workers seem to think that because they are working at the sea-side they should use the most disreputable and obsolete microscopes and lenses. Nothing is farther from the truth. If one is seriously engaged with the embryology of marine animals one must study the living eggs. The best optical equipment possible is therefore none too good. Accordingly I use the finest apochromatic lenses and compensating oculars procurable. A good darkfield condenser and a plankton condenser are valuable accessories for the study of the living sperm and eggs. For measuring, I use a screw micrometer with compensating ocular. Of course, such susceptible instruments need, particularly at the sea-side, special care.

Water immersion lenses

A good apochromatic water immersion lens is a valuable aid for the study of living eggs. However, a word of caution must be said: the lens coming in contact with sea-water must not be mounted in copper or other metal because by such mounts the sea-water is rendered toxic. Procure therefore a water immersion lens in a special non-toxic mount.

Frequently, a water immersion lens could with profit be used instead of an oil immersion. I have seen an experienced worker make observations on living *Amoeba proteus* and *A. dubia* under a 2 mm. apochromatic oil immersion lens of numerical aperture, 1.4. No such observation has value for, since these large protozoa are under great pressure, their behavior can not be normal. A 2.5 mm. water immersion lens with numerical aperture 1.25 would be more useful in such cases.

Other Accessories

A good stop-watch is indispensable for experiments that run for seconds only and for timing changes that endure for fractions of a second. When, as is often necessary, two stop-watches are demanded, they should be as closely synchronized as possible. I frequently use also an electric timing device. This need not be expensive for several reliable makes are now available at low cost.

Bolting-silk with mesh of various sizes is most useful. As shown beyond I use it for various purposes. Cheese-cloth, scrim, gauze and "bird's eye" cloth should be at hand; the first three named for straining eggs, the last named for cleaning slides and cover-slips. Lens paper is useful for experiments on the effects of pressure on eggs.

Literature

Just, E. E. 1915*a*. The morphology of normal fertilization in *Platynereis megalops*. **Journ. Morph., Vol. 26.**

———. 1915*b*. An experimental analysis of fertilization in *Platynereis megalops*. **Biol. Bull., Vol. 28.**

———. 1928. Initiation of development in the egg of *Arbacia*. V. The effect of slowly evaporating sea-water and its significance for the theory of auto-parthenogenesis. **Biol. Bull., Vol. 55.**

———. 1929. The production of filaments by echinoderm ova as a response to insemination, with special reference to the phenomena as exhibited by ova of the genus *Asterias*. **Biol. Bull., Vol. 57.**

II

NORMAL DEVELOPMENT

For the worker who aims to follow the normal development of a marine egg in the laboratory, the primary consideration is the assurance that the processes which he observes are the same as those that occur in nature. Descriptive embryology built upon laboratory observations stands only if one can assume that the stages observed represent faithful reproductions of those occurring in the state of nature. Thus, for the descriptive embryologist the normality of the object and the knowledge of this quality are most important. The acquisition of this knowledge is a task of no mean proportions because in no small degree is the work in the laboratory on descriptive embryology an experimental one since, in the best circumstances even, marine eggs are under not natural conditions.

The task of the experimental embryologist is still more onerous. He aims to make his experiments in such wise that they are capable of being repeated not only by himself but by others, reducing to the lowest limit the personal factor which enters so largely in biological investigations. He is, or should be, therefore, vigilant in repeating an experiment to reproduce all the circumstances attending it: working with the same volumes of eggs and of sperm in the same volume of sea-water of the same quality and at the same temperature. Variations in these factors being themselves experimental must be rigorously controlled. Also, for the experimental embryologist are the normality of the egg and the recognition of this quality basic prerequisites.

The basis and the control of any experiment is the perfectly normal egg; the worker must know therefore what is a good egg. The best source for this knowledge lies in the most thorough acquaintance of the normal egg in its normal surroundings. Whenever possible the normal development of the egg in nature should be followed.

Whenever this is not possible, the experimental embryologist should think twice before he undertakes laboratory work on the egg—especially if he can not procure a sufficient number of stages from natural surroundings with which to compare those that he obtains in the laboratory. The forms whose eggs I treat in this book have been studied in nature. The literature on them thus furnishes a guide as to their peculiar habitat, geographical and seasonal distribution, breeding habits, etc.—a guide which introduces the experimenter more quickly to the flavor of natural history, the pleasurable appetizer of biological work.

To be sure there are numerous instances on record that attest the fact that life-histories of animals have been ascertained only through observations in the laboratory: those of many nereids, of species of syllids, etc., phases of which were first described as distinct species. Also, are there forms, as for example, *Balanoglossus*, whose early life-histories up to now we know only as they run in the laboratory. These instances do not minimize the value of my point, namely, that the experimental embryologist should as far as possible know his animal personally and directly through work in the field, never resting content to become what Kropotkin in another sense denominated a "desk-biologist."

In most cases it is not an insurmountable task for the worker to collect his own animals. Indeed, he must often collect them himself because the sensitive nature of some animals demands extreme care in handling after capture and the greatest expedition in transport from collecting grounds to the laboratory. If one wishes to observe the fertilization-reaction in the egg of *Amphioxus*, one would best do this immediately upon capture of the animals because it is difficult to obtain shedding animals in the laboratory. Lwoff's old observation on this point I was fortunate enough to repeat in Naples. (Lwoff, 1892; Just, 1939; see also Orton, 1913–1915.) In the region of Roscoff, *Echinocardium caudatum* is extremely abundant but the animals even with greatest care are injured during transport to the laboratory. At Woods Hole, *Echinarachnius* if not properly cared for after having been dredged soon deteriorates. One may readily recognize deterioration in this animal because its

test contains a pigment which is a natural indicator: animals whose color has changed from red to green are moribund; if the water that contains them is green, the animals are deteriorating.

For the experimental embryologist, knowledge of the breeding-habits is indispensable for his work. Where these are periodic—as is true for many nereids, for example—his task becomes simple, as will be shown beyond. If the animals shed their gametes at low or high tide, this too is valuable to know. The late Dr. Gilman A. Drew made available for laboratory use the egg of *Cumingia* by learning that these clams brought to the laboratory in sand shed very quickly when isolated in dishes of sea-water. In many species of starfish the animals congregate for breeding so that fertilization occurs soon after the discharge of spermatozoa and eggs.

Many animals, especially those living at great depths of the sea, can not with profit be collected by the worker himself. Here he must depend upon the collecting staff, the dredging apparatus and boats of the laboratory. However excellent the apparatus for the collecting may be, the factor of prime importance is the collectors. If these be inefficient or untrustworthy, the experimentalist will suffer because of the bad condition of the animals furnished him. The collecting staff is thus very important for successful experimentation. A loyal and devoted collecting staff is of inestimable value to the reputation of a marine laboratory; one given to sabotage, preferring some investigators to others, is a disgrace. Here the investigator is not responsible for the poor results he may obtain; he is the victim of an inefficiency that is often not known to the laboratory authorities.

Whenever the experimental embryologist is dependent upon others for the collecting of breeding animals or of gametes, he must be confident that the collecting is properly accomplished. He should be sure that he has freshly collected organisms or cells; if these be aged, he has now to reckon with age as an additional complicating factor in his experiments. It would be difficult, I think, to over-emphasize this point.

In the first place, most marine animals withstand transport very badly especially during their breeding seasons; some withstand it not at all. Bonnevie reported results obtained on eggs

from *Membranipora pilosa* that had been shipped to her; these eggs she claimed were normal. I was never able to obtain viable eggs of this species at Naples except from freshly collected animals. The larger marine teleosts with few exceptions deteriorate rapidly after capture during the breeding seasons. In the case of the gametes themselves viability may diminish even more rapidly. A moribund egg is fit only for work which aims to study death changes and is valueless for experiments since it is incapable of giving us the necessary normal control. When therefore eggs and sperm are transported, they may be used only on the basis of sure knowledge that they have not suffered. The rule of paramount importance for the experimental embryologist is to use animals freshly collected and only from these, eggs and sperm freshly deposited or removed.

On the basis of his knowledge of development in the field, the experimenter can soon learn to know whether or not the development in the laboratory is normal. This knowledge, I repeat, is the *sine qua non* of all experimental work in embryology. For not only is normal development the main object of study, but it also represents the control of experiment and thus determines the quality of interpretation. Therefore, the investigator should, however laborious and long-enduring the task, follow the development of the egg in the laboratory and know that this is normal, before he begins to set up an experiment.

In the laboratory I have carried eggs of *Platynereis megalops* through to sexual maturity and from the eggs and sperm obtained reared a second and from the second a third generation of worms. At Naples I have reared *P. dumerilii* to sexual maturity; at Woods Hole, *Nereis limbata*. *Ciona* I have reared to sexual maturity (Naples). At Roscoff I have reared *Perinereis cultrifera* from eggs fertilized in the laboratory which reached a length of 4 cm., when I had to abandon the culture. In addition, I have reared to adult condition several species of sponges (Naples), hydroids (Naples), *Echinarachnius* (Woods Hole), *Asterias forbesii* (Woods Hole), *Diopatra* (Woods Hole).

Method for Diatom-Culture

Perhaps the greatest difficulty in rearing animals from eggs fertilized in the laboratory is feeding them. Several methods for

culturing diatoms to feed animals are now available. The most used is Allen's modification of Miquel's for culturing *Nitschia*. (Allen, 1907–1910; Miquel, 1890–1893; 1907.) My own method (Just, 1922) however is much simpler and in my experience superior. This follows:

At the beginning of the season, mud is taken from eel grass (*Zostera*) together with animal and plant life. This is placed in jars containing sea-water equal in amount to that of the mud. The jars are then covered with glass plates and set aside in subdued light. In a day or so all metazoa—worms, crustacea, ascidians, etc.—are dead. After a period of putrefaction the culture purifies itself and a rich growth of diatoms is apparent. It is a good plan to start several such cultures at intervals of five to ten days.

From the stock cultures thus prepared diatoms are removed, suspended in filtered sea-water, and strained through bolting silk. The diatoms that pass through the bolting silk are placed in the dishes containing the larvae. As the larvae increase in size and vigor, food is added in greater quantities.

With this method I have raised *Platynereis* embryos to adult worms in one-half-gallon Mason jars kept *tightly sealed* and never once opened during ten months.

My experience in rearing marine invertebrates from eggs—*Asterias*, *Arbacia*, *Echinarachnius*, *Nereis*, *Platynereis*, *Pectenaria*, *Diopatra*, *Chaetopterus* and *Mytilus*, etc.—indicates that the most essential point is to know when to begin feeding. In general, food must not be given until the larvae have used the oil and yolk present in the eggs. In all forms in which the oil is well defined, the time for feeding is readily ascertained. Where, as in echinoderms, the oil is not so clearly marked, one must make a few trials which will indicate the proper time for the introduction of the food.

Indicia for Normal Development

Once the worker has assured himself that he has normal eggs by having obtained normal development from them, he can soon learn to distinguish from the eggs themselves what their development will be. This knowledge will not only save time but also facilitate his work. If it is possible for him to know in a few

minutes by trial inseminations of the eggs that they will develop under laboratory conditions in a fashion that closely parallels, if indeed it is not identical to the development in nature, he can then set up his experiment on the basis of a sure knowledge concerning the normal condition of the cells at the outset. It is apparent that such information is highly valuable: it at once removes the uncertainty that attends experiments on many living cells whose physiological condition is obscure. For some species of eggs I have established such criteria from normal development.

In echinid eggs

I have found for eggs of echinids at Woods Hole, Naples and Roscoff, that one can quickly ascertain the degree of their normality by following their behavior at insemination and after very simple experimental treatment. (Just, 1928*b*.)

1. All echinid eggs that I know will, if they are in optimum condition, separate their membranes after insemination with fresh and active sperm-suspension at so uniform a rate that in a dish of a hundred eggs under the microscope one can observe that all eggs separate their membranes at almost the same instant. If the membranes do not separate fully, becoming equidistant from the eggs at every point of the surfaces, one may be sure that the eggs are not normal. Eccentric membranes, partially separated membranes and such that are slow in separating indicate that eggs or sperm are in poor condition.

Use fresh sperm! Perfectly normal eggs may give poor membranes upon insemination with sperm in poor condition.

2. Unfertilized eggs of echinids, most sharply those of *Arbacia*, exposed to distilled water separate their membranes while in the dilution in 15 seconds if they are in optimum condition. If they fail to separate membranes or separate them more slowly, they should be rejected for experimental work. Here lies the possible explanation of the failure of workers to obtain membrane-separation in echinid eggs exposed to distilled water as first reported by Schücking. I have made innumerable observations on the effect of extremely dilute sea-water on unfertilized echinid eggs always with the same result: if they do not separate their membranes, they are in poor physiological

condition. Although eggs from the same female may be fertilized, their development eventually reveals that the eggs are subnormal. In passing I may say that eggs with separated membranes due to the action of distilled water never develop beyond the monaster stage. This method therefore is not one for inducing experimental parthenogenesis.

3. A third index for normality of the echinid egg is furnished by the response of the eggs to exposure to distilled water 30 to 40 seconds after insemination, that is, during the process of membrane-separation. This response varies with the species of egg. The egg of *Echinarachnius* thus exposed, if normal, breaks down within sharply 15 seconds. The egg of *Arbacia*, on the other hand, if in normal condition, shows the effect of exposure only in the blastula stage.

4. Years ago Loeb (1894) found that eggs of *Arbacia* exposed to dilute sea-water some time after fertilization tend to protrude through the ruptured membranes forming extra-ovates which develop into twins with or without separation of the two egg-fractions. European workers who endeavored to repeat Loeb's observation on European species were not always successful. I have found, however, that one can produce such extra-ovates in various species of sea-urchins. My method is simpler and I think more exact than Loeb's. I expose the eggs, of *Arbacia*, for example, to distilled water 3 minutes after insemination. If the eggs are in best condition, 90 to 100 per cent. of them protrude through the ruptured membrane. Eggs now returned at once to normal sea-water develop into twins which may remain attached or become separated. This capacity for the formation of extra-ovates is an excellent criterion for the eggs' normality.

I speak somewhat at length concerning these criteria for normality in the echinid egg, because very recently my experience at Roscoff has shown me again how valuable they are as diagnostic for the eggs' physiological condition.

In nereid eggs

In forms like the nereids, that have a heteronereid phase, swimming at the surface of the sea at definite phases of the moon, nature provides us already with an index of normality, for only

mature worms with fully ripe sexual products swim. Thus one may be sure of the perfect condition of the gametes. This is the great advantage of working with the egg of these and similar forms.

If one desires to carry on experiments that demand normal larvae, the distribution of the oil drops furnishes a criterion of value for three species of *Nereis:* I found that the normal trochophore shows one and only one oil drop in each of the four cells of the gut. (Just, 1922*b*.) The egg of *Perinereis* at Roscoff, more hardy than that of any other nereid egg known to me, is in this respect similar to *Nereis* eggs: its larvae show, if they are normal, the same distribution of the oil drops.

In Asterias eggs

The eggs of *Asterias* are normal only if after having come into sea-water their germinal vesicles fade at the same rate. This rate depends upon temperature, provided the eggs are suspended in sufficient volume of sea-water. Contrary to the belief of many workers, this egg is in the optimum condition for fertilization after the break-down of the germinal vesicle and before the extrusion of the first polar body. Work done on the egg of *Asterias* after complete maturation is work done on an egg no longer in optimum condition.

In other eggs

Echinid eggs represent one, and nereid eggs represent the other of the extremes with respect to the time when in their development eggs are fertilizable. That is, echinid eggs are fertilizable only after complete maturation and nereid eggs are fertilizable only in the germinal vesicle stage. For eggs like those of *Chaetopterus*, whose germinal vesicles break down after the eggs come into sea-water, the criterion for normality obviously is the appearance of the first maturation spindle. Undoubtedly, one could ascertain experimental tests comparable to those detailed above which would serve as valuable indices for normality. These have yet to be devised. Eggs fertilizable in the stage of second maturation, as for example those of *Amphioxus*, also would reveal criteria by which one could establish prognostics of subsequent normal development.

So far as I know the only information we have concerning *Amphioxus* is that, according to Hatschek (1882), the eggs lose capacity for development very quickly: they develop normally only when shed into water in which the males have previously discharged their spermatozoa.

Polyspermy

In general, all normally monospermic eggs that I have studied, are never polyspermic if they are in optimum condition. The reported polyspermy in eggs of *Membranipora* (Bonnevie, 1907) I could not during a year's study at Naples confirm (Just, 1934.) The alleged polyspermic fertilization of *Pedicellina* (MacBride, 1914) is due to misunderstanding of Hatschek's report (1887) that he observed spermatozoa in the perivitelline space. For any of the eggs named in the foregoing, and for all those specially treated in this book, polyspermy is a sign of low vitality.

Tempo of Development

If one takes a suspension of *Arbacia* eggs known, by means of one of the criteria discussed above, to be perfectly normal and inseminates them with a freshly prepared sperm-suspension and at the same time takes a suspension of *Echinarachnius* eggs which are also perfectly normal and inseminates these, one can observe the development of these two species of eggs under identical conditions with respect to temperature, volume of sea-water, etc. One learns that the rate of development in the two species is not the same. The larger, more transparent *Echinarachnius* egg develops more slowly. In like manner, one can make a comparison between eggs of *Nereis limbata* inseminated at the same instant that a *Platynereis megalops* lays its eggs. The larger, more transparent *Platynereis* egg reaches first cleavage before the egg of *Nereis*. If one goes farther afield and compares eggs of distantly related species one notes again that under uniform conditions the tempo of development varies with different eggs. It is apparently not possible to correlate these differences with any visible characteristic, as size or transparency of the eggs. The problem of the tempo of development is one worthy of more attention than it has received.

Tempo of development as criterion for normality

The chief value of knowing the tempo of normal development for a given egg lies in the fact that the investigator can quickly ascertain at every stage departures from the normal. As the rate of membrane-separation mentioned above serves in the case of certain eggs as a diagnostic of their normality, so the rate of cleavage and of subsequent phases gives information concerning normality. Any departure from what is known to be the normal rate of development of eggs, whose fertilization has been normal, points to some change induced by the observer.

Temperature

It frequently happens that a worker begins with perfectly normal eggs which deteriorate during the course of development because of abnormal temperature conditions. Some fluctuations are bound to occur, but these should be reduced as far as possible. Further, unless eggs are normally found in sea-water of low temperature, *they should never be kept in the cold* except for the expressed purpose of investigating the effect of low temperature on an egg whose normal habitat is at higher. I have known many investigators who have kept their animals on ice over night in order to delay shedding. This practice, I think, can not be too severely condemned. Finally, in making observations on the normal development of an egg, from the container in which the eggs are kept the worker should take samples throughout the course of the observation and should not be content to observe only those eggs transferred to a small glass-container at the beginning of the course of observation. In the small watch-glass or on the microscopic slide, the eggs suffer from change in temperature and may also be affected by increase in salinity due to evaporation. This same consideration holds for experimenting on eggs that are in too small a volume of sea-water.

Evaporation

Not only should changes in temperature be guarded against, but also changes in salt-concentration of the sea-water brought

about by evaporation. Dishes containing eggs should be kept covered. More than once results have been reported as due to an experimental treatment which actually were brought about by evaporation of the sea-water containing the eggs. I have called attention to such cases. Two such alleged effects I have disproved. (Just, 1928*a*; 1929.)

Seasonal Changes

When we speak of the breeding season, we do not mean that the gametes are in exactly the same condition throughout this season. For every animal there is variation during its breeding season with respect to the quantity and to the quality of its sexual products. For example: during the period in which one can obtain ripe eggs and sperm from various heteronereids, there is a time when the eggs and sperm can be obtained in maximum abundance. At Woods Hole, the heteronereid form of *Nereis limbata* appears at the beginning of the breeding season in small numbers, which later rise, then taper off, so that in September the number of animals captured is very small. The quality of the gametes of this worm, however, remains the same, as has been mentioned above. In other forms, as echinids, the sexual products vary also in quality. At the beginning of the breeding season of any named echinid the number of fully matured eggs is small. This number increases as the season progresses. There comes an optimum period when the eggs and sperm are qualitatively in best condition. This is followed by a period during which the gametes are of poorer quality. In this time the eggs are not so amenable to experimental treatment. Many so-called over-ripe eggs are now obtained which often fail to fertilize. (Just, 1922*a*.) The worker in experimental embryology, therefore, would do well to know these seasonal variations and to plan his experiments accordingly. For any named species, the breeding season varies with the locality. For example, at Roscoff the breeding season for *Strongylocentrotus lividus* extends farther into the summer than at Naples

To many a worker who has obtained results in default of following these simple directions, I may seem to be pedantic

in emphasizing them. Nevertheless, I am strongly convinced that the most essential prerequisite for clear-cut and valuable experiments on marine eggs is strict adherence to every one of these suggestions. An experiment which in the least way is clouded by uncertainty concerning the normal process surely has little value for the elucidation of this.

Literature

Allen, E. J. and E. W. Nelson. 1907–1910. On the artificial culture of marine plankton organisms. **Journ. Marine Biol. Assoc., Vol. VIII, N.S.**

Bonnevie, K. 1907. Physiologische Polyspermie bei Bryozoen. **Jena Zeitschr. Naturw., Bd. 42.**

Hatschek, B. 1877. Embryonalentwicklung und Knospung der *Pedicellina echinata*. **Zeitschr. wiss. Zool., Bd. 29.**

———. 1882. Studien über die Entwicklung des *Amphioxus*. **Arb. Zool. Inst. Wien. 4.**

Just, E. E. 1922*a*. Initiation of development in the egg of *Arbacia*. III. The effect of *Arbacia* blood on the fertilization-reaction. **Biol. Bull., Vol. 43.**

———. 1922*b*. On rearing sexually mature *Platynereis* megalops from eggs. **Amer. Naturalist, Vol. 56.**

———. 1928*a*. Initiation of development in the egg of *Arbacia*. V. The effect of slowly evaporating sea-water and its significance for the theory of auto-parthenogenesis. **Biol. Bull., Vol. 55.**

———1928. *b*. Initiation of development in *Arbacia*. IV. Some cortical reactions as criteria for optimum fertilization capacity and their significance for the physiology of development. **Protopl., Bd. 5.**

———. 1929. Initiation of development in the egg of *Arbacia*. VI. The effect of sea-water precipitates with special reference to the nature of lipolysin. **Biol. Bull., Vol. 57.**

———. 1934. Fertilization in *Membranipora pilosa*. **Carnegie Year Book, 1934.**

———. 1939. The biology of the cell surface. **Blakiston, Philadelphia.**

Loeb, J. 1894. Über eine einfache Methode, zwei oder mehr zusammengewachsene Embryonen aus einem Ei hervorzubringen. **Arch. ges. Physiol., Bd. 55.**

Lwoff, B. 1892. Über einige wichtige Punkte in der Entwicklung des *Amphioxus*. **Biol. Centralbl. 12.**

MacBride, E. W. 1914. Textbook of embryology. **Macmillan, London.**

Miquel, P. 1890–1893. De la culture artificielle des Diatomées. **Le Diatomiste I.** (**Le micrographie Préparature V, 1897.**)

Orton, J. H. 1913–1915. Notes on experiments on rearing *Amphioxus*. **Journ. Marine Biol. Assoc., Vol. X, N.S.**

III

METHODS FOR HANDLING EGGS AND SPERM IN THE LABORATORY

Before I take up the discussion of individual species of eggs and spermatozoa, I should like to speak briefly on some points of general application.

General Considerations

A convenient classification of eggs is based on the stage of maturation at which they are fertilizable. In this wise, all animal eggs fall into four classes: those fertilizable before maturation (in the stage of the intact germinal vesicle); those fertilizable during first maturation; those fertilizable during second maturation; and those fertilizable after complete maturation. Classes 1, 3 and 4 offer no difficulty for fertilization in the laboratory, because the eggs are shed in the fertilizable stage. Class 2 comprises some species of eggs which are shed with the germinal vesicle intact whose break-down is stimulated by the sea-water. Here one must wait with fertilization until this break-down has occurred. Some other eggs of this class are shed with the first maturation spindle forming or formed; at this time they may at once be fertilized. In handling eggs and sperm one must ascertain to which of these classes the species of eggs employed belongs. The addition of sperm to eggs not yet in their fertilizable stage may often keep the eggs from attaining this stage. (On this point see the discussion on the egg of *Asterias* below.) In other cases, as for example in eggs of echinids, although the spermatozoa induce changes in the eggs with intact germinal vesicle or in stages of maturation, these eggs never develop.

Another general point concerns the aging of eggs as they lie in sea-water. This varies with the species. Some eggs, as those of *Platynereis megalops* and of *Amphioxus*, lose fertilization-capacity very shortly after having come into sea-water.

Others, as those of *Nereis limbata* and of *Arbacia*, remain fertilizable for a longer period. Eggs which withstand residence in sea-water, lose this resistance at higher temperatures and after repeated washing with sea-water. One must, therefore, know the capacity of the egg which one uses to resist the action of sea-water. *It is best to use eggs freshly shed or removed from the animals and to avoid the use of stale eggs.* Staling of eggs is itself an experimental procedure used both in straight and especially in cross-fertilization.

In general, sperm-suspensions should be freshly prepared because spermatozoa, especially in attenuated suspension, lose viability in sea-water. Even actively motile sperm may be of low fertilizing power. Where sperm are inactive, one must determine the cause of this inactivity. If it is due to immaturity, the sperm-cells will not fertilize eggs. Only mature spermatozoa are capable of fertilization. Where sperm are removed from the males, one must establish whether or not they are ripe. Unripe sperm often appear in bundles. Spermatozoa of squid are in spermatophores; these should not be removed from the animal until the moment when fertilization is to be made. At this time the spermatophores are brought into sea-water which induces the discharge of the spermatozoa. Decapod (Crustacea) spermatozoa explode singly in sea-water, as Kolzoff has shown.

Notes on Special Forms

There follow now some notes on particular forms. This emphasis on the eggs of these species does not of course imply that all experimental work should be done on them. On the contrary, some problems in experimental embryology can be best attacked by study of eggs not detailed beyond. I do not mention eggs of sponges and coelenterates; nevertheless, they are admirable for certain researches and ought be studied more. In certain flat-worms one can follow under the microscope the process of egg-laying, the escape of the egg from the ovary, the spinning of yolk around it, and the secretion of the shell that encloses it—a fascinating observation. These eggs also have been too greatly neglected by experimental embryologists. Nevertheless, a certain advantage adheres in using for experi-

ments eggs and sperm that are abundant and easily available. If in addition such eggs are universally popular among experimental embryologists, we ought to have some simple directions for handling them.

Arbacia

The worker may obtain eggs and sperm of *Arbacia* in optimum fertilizable condition by one of several methods: (1) allowing the animals to shed; (2) cutting carefully around the peristome, without injury to the gonads, or removing the spines, either of which stimulates shedding; and (3) cutting around the equator of the test and removing the ovaries to 250 cc. of seawater, the testes to a dry watch glass.

(1) Freshly collected ripe *Arbacia* readily shed their sexual products. Indeed, this may be a nuisance if the worker has among several such animals in a tank one that starts shedding. I have frequently observed an animal on the side of a tank begin shedding; next, the adjacent one sheds and so on around until soon from every ripe animal eggs or sperm stream forth. Similarly, I have found thick mats of eggs lodged among the spines of females on the bottom of the tank. Elsewhere (Just, 1922) I have commented on the fact that I have taken perfectly normal eggs in various stages of development from among the spines of living females. I have also collected, from the piling of the wharf opposite the Crane building at Woods Hole, *Arbacia* that shed before I could get them to the laboratory, a distance of about one hundred yards.

These shed eggs are of optimum fertilizability except toward the end of the breeding seasons (Just, 1922). Since one could scarcely depend on shed eggs for one's experiments, one must use other methods for obtaining eggs in optimum condition. I therefore suggest the following methods.

(2) Eggs shed as the result of stimulation through injury to the animal, e.g., by cutting carefully around the peristome—without puncturing the ovaries—or by removing their spines, are by no means inferior to eggs normally shed, as their percentage and normality of development reveal. The animals thus stimulated should be placed aboral surface down in clean dry Syracuse watch-glasses, and the eggs so obtained ("dry

eggs"), free from perivisceral fluid, if from intact ovaries, should be removed as they exude to 250 cc. of sea-water. Sperm thus obtained ("dry sperm") should be kept undiluted and covered until needed for insemination. This is important because the fertilization power of *Arbacia* sperm-suspensions falls off with dilution (Lillie, 1915; Cohn, 1917). For insemination a thin sperm-suspension is freshly made. I use one drop of "dry" sperm in 10 cc. of sea-water, from which I take two drops to inseminate eggs in 250 cc. of sea-water.

Like those normally shed, these eggs are invariably free from perivisceral fluid and except at the end of the breeding season are of high fertilization capacity. A slight accidental puncture of the ovaries means contamination by the body fluid, which seeps into the ovary and through the genital pores with the extruded eggs. In such an event the eggs must be washed free of the perivisceral fluid which inhibits fertilization. One should therefore make trial inseminations on samples taken from eggs suspended in 250 cc. of sea-water. If 95 to 100 per cent. of the eggs fertilize as shown by the number that separate normal membranes, they are good. If the per cent. and quality of membrane-separation be low, the worker should wash the eggs again and make another trial insemination on them. This procedure should be repeated during an hour after the eggs have first been suspended in sea-water, until normal fertilization in close to 100 per cent. of the eggs is obtained.

The inhibitory action of the perivisceral fluid to the fertilization of *Arbacia* eggs was first shown by Lillie. The reader should consult his book, "Problems of Fertilization," for references. I have confirmed Lillie's findings on eggs of *Arbacia* and on those of *Echinarachnius* not only for fertilization, but also for experimental parthenogenesis. (Just, 1919; 1922.)

(3) I dare say that the most common method of obtaining *Arbacia* eggs is that of removing the gonads directly to sea-water. By this method the eggs most certainly suffer from contamination by perivisceral fluid. One may rid them of this as follows:

Open the animal with a circular cut aborally through the test slightly above the equator. Discard the oral part of the animal. Now invert the aboral part and so drain off and dis-

card the perivisceral fluid. Next, either place it in a clean Syracuse watch-glass so that the eggs exude through the genital pores, or carefully remove the ovaries to 250 cc. of sea-water into which the eggs will fall freely from the ovaries. In the latter case, strain the eggs free from débris by putting them through cheese-cloth, previously soaked in running sea-water after having been washed in fresh water.

If necessary, wash the eggs four times by decanting the sea-water above them as soon as they have settled, adding very gently sea-water in an amount equal to that removed. Again the washing should not take more than one hour. After each washing, test the eggs by trial insemination on samples. If after the fourth washing the trial insemination does not yield close to 100 per cent fertilization, discard these eggs and use the eggs from another female that yield practically 100 per cent fertilization.

I find it worth while to open several females, selecting the eggs from the best. I never mix the eggs from several females. One point the worker must remember: *Arbacia* eggs are not "C. P." chemicals that give the same results day in, day out. Too many variables enter: the time in the breeding season, the freshness and vigor of the animals—which depend upon the length of time they have been in the live cars, after having been collected—the fullness of the gonads—which depends to a great extent upon the collecting grounds from which the animals come during a given lunar period—the abundance of the "blood inhibitor" present, temperature, etc.

In passing, I may note that I also use *Echinarachnius* to feed *Arbacia*, thus restoring the sea-urchins previously in poor condition to a high degree of excellence.

Echinarachnius

The egg of *Echinarachnius* is one of the most beautiful in the Woods Hole region. It is larger than that of *Arbacia* and possesses less pigment. *En masse*, the eggs are of a red hue because of the pigmented jelly hulls that enclose them; when this jelly is removed, they are a pale yellow—lighter than an equal mass of *Asterias* eggs. Their color is due to chromatophores

which the worker may have some difficulty detecting. They can, however, be found with ease in the later stages of development.

Echinarachnius does not stand up well under adverse treatment. One should therefore be sure that one has animals in prime condition. Fortunately, this is readily ascertained.

The normal intact animal is brownish red and discharges no color into the sea-water. If the animal be injured locally the point of injury becomes green, and the sea-water above it also. One may prove this by scraping a part of the test of an animal in perfect condition. Very quickly as the alkaline sea-water penetrates, the injured spot turns green. If one pours on to an intact animal of normal color N/10 NaOH, it turns green, but if one uses N/10 NHOH instead, the animal takes on a purple hue. Only animals of the normal color should be used. I find that the red pigment in the egg is also a natural indicator. (For color indicators in other echinid eggs see Crozier, 1916.)

The animals are best kept on a clean concrete sea-water table. Though I have used *Echinarachnius* through August, I prefer to work on them earlier because then they come to the laboratory in better condition. This is largely due to temperature; the animals are not at their best when crowded in the tubs after having been dredged from deep water during warmer days. This is shown by the rapidity with which the sea-water in the tubs is charged with the green color. At all times freshly collected animals, properly cared for after collection, are best.

I have frequently obtained *Echinarachnius* eggs normally shed. As in the case of *Arbacia* the shedding may be induced by injury—cutting the lantern or around the margin of the animal. For obtaining eggs of optimum fertilization capacity from the ovaries, these directions should be followed:

Cut around the margin of the animal, remove and discard oral portion. Place aboral portion (with the outside down) in a clean, dry, Syracuse watch-glass. If the animal is ripe, sperm (or eggs) will ooze from the gonads. Allow an opened male to remain until you are ready for insemination. From the female very carefully pipette off the eggs to 200 cc. of clean sea-water. The sea-water in which the eggs are suspended should be clear and not opalescent or milky through the presence of perivisceral

fluid. Allow the eggs to settle. Pour off the supernatant sea-water and very carefully add sea-water up to the original volume employed. Now strain the egg suspension through clean, washed cheese-cloth wetted with sea-water. The eggs are now ready for use. If the time consumed in opening the animal and preparing the eggs amounts to more than one hour, open some more animals until you get a good male, discarding the others, in order to have perfectly fresh sperm. Inseminate the eggs as you would those of *Arbacia*.

Strongylocentrotus, Sphaerechinus, Echinus and Echinocardium

Methods for *Strongylocentrotus*, *Sphaerechinus* and *Echinus* are the same as those for *Arbacia*.

For *Echinocardium* I proceed as follows: The fragile test is opened carefully on the oral side and the portion of the test thus cut is gently removed. If the eggs exude from the genital pores, the animal is placed in a dry watch-glass. If, instead, the eggs ooze from the ovaries, they are removed to clean sea-water. Opened males are placed in dry watch-glasses and the sperm pipetted off to sea-water to make up a sperm-suspension just before insemination is planned. The egg of *Echinocardium* is beautifully transparent and most favorable for observation during development. The animal, however, is very fragile and hence sensitive. It should be handled with utmost care.

Asterias

The eggs of *Asterias forbesii*, *A. vulgaris* and *A. glacialis* are most beautiful for many purposes, when properly handled; unfortunately they are greatly maligned by many workers. This is not the fault of the egg. The first essential is to get good animals with ripe gonads.

Asterias forbesii and A. vulgaris

When fully ripe the animals are heavy, their skin is soft and their arms bulging. I determine ripe animals by roughly estimating their weight, rejecting the lighter ones with firm brittle skin and narrow arms. Frequently, I have been able to select the ripe animals so exactly that when using eggs only,

I have taken but one specimen. The ovaries of a large ripe female will fill a dish of 200 cc. capacity.

Asterias shed eggs readily in the laboratory. In June, 1927, for example, I had a great deal of difficulty because of this fact. The first normally shedding male and female I ever used were kindly turned over to me in 1910 by Dr. John W. Scott. Since then I have recorded many observations on animals shedding in the laboratory. Every one of these was during full moon, never during new moon. The average worker would hardly care to await the chance of procuring normally shed eggs. This is indeed not necessary, since he can obtain eggs of optimum viability by removing the ovaries to sea-water.

In the interest of economy it is well to make a slight puncture in an arm close to the disc, and pipette off a few drops of cells from the gonads. The animal is not seriously injured thereby and its sex may thus be ascertained. The animals are best opened as follows:

Make a cut along the mid-dorsal line of each arm beginning at the tip and across the central disc. Bend back the flaps thus made and expose the gonads. If the animal be a ripe female with well filled ovaries, with forceps carefully remove each ovary with as little injury as possible and place it in at least 2000 cc. of sea-water in a large flat-bottom dish. *Do not cut up the ovaries; the eggs will exude freely.* When the eggs from the blunt end of the ovary have streamed out into the sea-water, remove the ovary, for you now have the best eggs. Stir the water gently and then allow the eggs to settle. (They settle more slowly than those of *Arbacia*.) After the eggs have settled pour off the sea-water and add an equal volume of sea-water. Note under the microscope the break-down of the germinal vesicle. If the eggs are in good condition practically not a single one will show an intact germinal vesicle.

If the animal opened proves to be a male, *cut through one arm only.* Snip off a small bit from the blunt end of the testis and place this in 200 cc. of sea-water. The sperm, contrary to the somewhat current notion, are highly active, although not as much so in concentrated suspensions as those of *Arbacia*.

I venture the opinion that workers experience difficulty in handling eggs of *Asterias*, even when they have animals in perfect

condition, for three chief reasons. First, they often crowd the eggs in a small volume of sea-water. Eggs placed directly from the ovaries in very little sea-water often fail to maturate; this failure is an effect of CO_2. Butyric acid and insemination also inhibit maturation. On the other hand, maturating eggs are highly susceptible to CO_2, butyric acid, and elevation of temperature because all of these agents initiate development. Shaking matured eggs, as Mathews (1901) has shown, causes them to develop. In this last case my own observations indicate that CO_2 here also plays a part.

Secondly, workers, because they do not use sufficient care in opening the animals and removing the gonads, too frequently contaminate the eggs with perivisceral fluid or tissue extracts. *Asterias* eggs are the most sensitive that I know. The worker will obtain infinitely more constant results if he treats this egg with respect. In addition, he would save time and would avoid the needless destruction of animals.

Thirdly, the practice of chopping up the ovaries for obtaining eggs mitigates against securing a high per cent. of normal development. By this method many young ovocytes are released whose germinal vesicles are not stimulated to break-down when the eggs are brought into sea-water. In this case, one may often count more eggs with intact germinal vesicles than those whose germinal vesicles are breaking down.

The worker can prove to his own satisfaction that the method for handling *Asterias* eggs which I have outlined above is a good one. First, let him take shed eggs and inseminate them. Next, inseminate eggs from the ovaries of the shedding female as outlined above. Finally, let him now cut up the ovaries and inseminate the eggs thus obtained. He will find that while the eggs of the first and second lots are about the same, as they reveal by their high per cent. and normality of development through the bipinnaria stage, the eggs from the cut-up ovaries are distinctly inferior in both respects.

Asterias glacialis

The method for *Asterias forbesii* and *vulgaris*, I have used with success at Naples for *A. glacialis*. In January, 1929, I obtained my first lot of shedding *A. glacialis* at Naples.

One final word which concerns all these species of starfish: the optimum moment for fertilization comes during the stage of the eggs' first maturation. Eggs inseminated in the stage of the intact germinal vesicle never leave this stage. After separation of the first polar body, fertilization capacity begins to fall off; although fertilization is possible even after complete maturation, neither it nor later development is normal.

Thyone

As far as I know, Pearse was the first to observe the shedding of eggs by *Thyone* in the Marine Biological Laboratory, Woods Hole. I have during several seasons obtained eggs in optimum condition for fertilization by allowing the animals to shed. For many workers this may not be a good egg because of its opacity. It has nevertheless some interesting points. *Thyone* is extremely abundant at Woods Hole.

Synapta

At Roscoff I have studied eggs of *Synapta* shed in the laboratory.

Nereis limbata

Unlike the forms that I have so far considered in this section, *Nereis limbata* is sexually dimorphic. The males are bright red anteriorly with white posterior segments, the females pale yellow or light green.

The animals are caught after sunset on certain nights, with a few exceptions, during the "dark of the moon" in the months of June, July, August and September. (Lillie and Just, 1913.) In Woods Hole, the most favorable locality for collection is the float stage in the Eel Pond back of the Supply Building. The worms appear swimming near the surface of the water about an hour after sunset. Attracted by the light of a lantern or an electric light (in Woods Hole, the float stage has been wired and two electric plugs are to be found in a box attached to the boat-shed) the worms are readily caught with a hand net. In general the swarming begins with the appearance of a few males swimming rapidly in curved paths in and out of the circle of light cast by the lamp. The much larger females then begin to appear,

usually in smaller numbers, swimming laboriously, frequently not coming to the surface of the water. Both sexes rapidly increase in numbers during the next fifteen minutes, and in the case of a large swarm, hundreds of males may be in sight at one time. The females are less numerous, though on one night I caught enough to fill a liter jar. A night's swarm lasts for an hour or an hour and a half. Each night from full moon to new moon, with certain exceptions, this scene may be re-enacted. And each night the females swarming are a new crop.

During the light of the moon, except for the first (June) run, when some animals may in certain seasons appear each night until the next swarming period (July, full moon), no *Nereis* swarm. And late in September if the nights be cold, they do not swarm throughout the dark of the moon. With these exceptions the swarming of *Nereis* corresponds to the four lunar cycles during June, July, August and September. Each run begins near the time of full moon, increases to a maximum during the succeeding nights, sinks to a low point about the time of the third quarter of the moon, then rises again to fall to extinction at or shortly after new moon. Thus, the curve of nightly numbers during a run is bimodal.

When a female appears, the males at once surround her, swimming rapidly in ever narrowing circles. In a short time, they shed sperm so that the water appears milky. The female sheds her eggs, shrinking in bulk and in so doing becomes a mere shred of tissue, sinks slowly from view, and dies.

The earlier work at the Marine Biological Laboratory, Woods Hole, on eggs of *Nereis* was done at night. The animals shedding when collected were placed in the same vessel, and therefore the eggs were fertilized then or soon after. For the early stages, one was obliged to begin one's observations at once.

The worms may, however, be kept over night without detriment. The animals should be collected singly and each female placed in a separate finger bowl. Three or four males may be kept in one finger bowl. If the animals are to be kept over night for work the next morning, the sea-water in which they were placed when captured should be renewed in the laboratory. *The sexes should never be mixed.* The finger bowls are covered and placed on the sea-water table with water flowing

around them. The practice of keeping the animals in a refrigerator can not be too severely condemned. The great excellence of *Nereis* for experimental work is that every swarming individual is always sexually mature, and contains no immature sexual cells.

Directions for obtaining eggs and sperm are simple.

To obtain eggs, wash an isolated female by placing her in clean sea-water, and snip her with sharp scissors. The eggs will pour forth quickly. Remove the cut animal, wash the eggs by pouring off the sea-water in which you had placed the worm and add an equal volume of sea-water. I use 250 cc. of sea-water. Now wash a male by placing him in 250 cc. of sea-water. Remove and dry him lightly and quickly on soft filter paper. Place in a clean dry Syracuse watch-glass and make a small cut about half way between head and tail, along the lateral border to avoid cutting the dorsal blood vessel. This gives you clean, "dry," sperm-suspension, free from blood. For an insemination add one drop of dry sperm to 10 cc. of sea-water. Of this sperm-suspension, use two drops to the eggs of one female.

Because of its almost clock-like precision of development, one could scarcely wish for a finer object than the egg of *Nereis*. If one does not get 100 per cent fertilization and almost perfectly uniform rate of cleavage, one's technique is at fault. The worker who believes in wide variability in the development of eggs from one female should study the eggs of *Nereis* properly collected and handled. Permit me to say that generalizations on the wide variability among eggs from a female of a given species are disproved if one uses animals in optimum condition and handles properly their eggs and sperm. *Nereis* when caught are always in optimum condition. The worker's results therefore depend solely on the methods he employs after collecting the worms. I make it a rule never to use *Nereis* that have been in the laboratory more than sixteen hours.

Platynereis dumerilii

Platynereis dumerilii may be obtained in the heteronereid phase at Naples throughout the year, as Ranzi has shown. (Just, 1929, Ranzi, 1931.) The methods for handling the eggs and sperm are the same as those given for *Nereis* except that the

animals in my experience tend to shed more readily. It is best, therefore, to experiment on these eggs on the night of capture. During the winter months, one can procure eggs and sperm by rearing in the laboratory worms collected in the nereid phase.

Chaetopterus

The sexes in *Chaetopterus* are distinguished by the color of the sexual elements located in the parapodia; the eggs are orange-colored, the sperm milky white. The sexes should be kept separate, each individual being placed in a separate dish under a gentle stream of sea-water.

The eggs are removed by cutting the parapodia containing them. The mucus present is removed by putting the eggs through cheese-cloth. The eggs are then washed and set aside for about fifteen minutes or until the first maturation figure in the metaphase reaches the periphery of the egg. If not inseminated, the eggs die in this stage.

Sperm are obtained by removing a posterior parapodium which is cut in 10 cc. of sea-water. If a drop of the sperm-suspension examinated under the microscope shows bundles of nonmotile cells, spermatids, spermatocytes and spermatogones, the animal is not ripe. Mature spermatozoa exude freely and are highly motile. Insemination is made as for eggs of *Nereis*.

Podarke

Treadwell (1902) has used the eggs of this worm. He collected them during the day and allowed them to spawn (at night). I have seen several hundreds of these worms swimming at the surface of the sea during *Nereis* runs. Because of their small size they are hard to handle when taken at night, for they are then shedding freely their eggs and sperm. If one wishes, therefore, to study fertilization, one should collect the animals during the day—from eel grass in the Eel Pond at Woods Hole.

Amphitrite

Amphitrite, which is very abundant in the Woods Hole region, breeds "within two days of the new and full moon" during the summer months. Shed eggs are always free from admixture of coelomic corpuscles and unripe eggs. Every egg

laid is capable of fertilization, but if one cuts up the animals to procure the eggs, one obtains mature eggs, inactive eggs and coelomic corpuscles. (Scott, 1906.)

The case of *Amphitrite*, one of the most interesting that I know, recalls the precision of the egg-laying in *Echinorhynchus*, where only fertilized eggs, among the fertilized and unfertilized which the uterine bell takes up, pass outside the female.

Phascolosoma gouldii

Phascolosoma eggs are best when laid. On one occasion only, in 1911, was I able to fertilize eggs obtained by cutting up the animals to procure gametes. Eggs and sperm thus obtained are mixed with innumerable coelomic corpuscles; shed eggs and sperm are free of them.

Phascolosoma vulgare and Ph. elongatum

At Roscoff, *Phascolosoma vulgare* and *Ph. elongatum* are best when shed. (For all three forms of *Phascolosoma*, see Gerould, 1907.)

Cumingia

Workers who use *Cumingia* are indebted to Dr. Gilman A. Drew, formerly assistant director of the Marine Biological Laboratory, Woods Hole, for the method of obtaining the eggs.

The animals are collected at low tide, kept in mud, protected from rise in temperature, and brought to the investigator as quickly as possible. They are placed under running water for about fifteen minutes or until wanted. Each animal is now transferred to a separate dish—the females in finger bowls containing about 200 cc. of sea-water, the males in dishes of about 50 cc. capacity. I distinguish the sexes by the color of the sexual elements seen through the shells: females show a salmon pink, the males a dead white color. If the animals are not disturbed, in less than half an hour they set free their eggs and sperm. I pipette the eggs off as soon as they are shed. Inseminations should be made from a thin sperm-suspension.

Ensis

The eggs of *Ensis* resemble greatly those of *Cumingia*. They may be obtained in abundance and with ease, especially

during July. Animals kept one to a dish shed rapidly. It is more convenient to use the smaller specimens placing them in finger bowls; larger animals usually shed more eggs but need larger dishes. The eggs are useless if taken from the ovaries. The animals are best kept in wet sand after collecting and should be protected from rise in temperature.

Mytilus

The eggs of this form can be obtained in very large numbers. They too resemble the eggs of *Cumingia*. For the worker who desires more eggs from one female than he can procure from *Cumingia* or *Ensis* for experiments similar to those for which he used *Cumingia* eggs, here is the animal to use. There is the drawback, however, that the animals must shed the eggs; eggs taken from the ovaries are impaired. The abundance of *Mytilus* makes it easy to get eggs throughout the breeding season. This egg was studied by Hertwig and more recently by Meves (1915). The reader should consult Field's monograph, "Biology and Economic Value of the Sea-Mussel, *Mytilus edulis*." (1923).

It is not at all difficult to carry this egg through metamorphosis. Indeed, *Mytilus* used to cause a great deal of trouble in the old sea-water tank at the Marine Biological Laboratory, Woods Hole. Larvae developed in such numbers that the mussels interfered with the water supply. I have had young clams in diatom cultures a year old; these had developed from veligers.

Mya

The eggs of *Mya* are obtained by allowing the animals to shed. At Woods Hole, eggs are plentiful during the summer.

Mactra

Unlike *Cumingia*, the eggs of *Mactra* are fertilizable in the germinal vesicle stage, thus resembling the eggs of *Nereis* and *Ascaris*. This makes it an interesting form for work on fertilization and experimental parthenogenesis. For the latter point especially see Kostanecki's papers (1904 and 1911).

Eggs of *Mactra* differ from those of *Cumingia* and of the other clams mentioned in still another way—they fertilize

readily if taken from the animal. There is no scarcity of *Mactra* in the Woods Hole region. This is a beautiful egg admirably suited for experimental work.

Pecten

Pecten is monoecious and, in my experience at least, seems to be self-fertilizable. In battery jars each of which contained a single individual I have repeatedly found fertilized eggs which developed into veligers. With suitable food one could doubtless carry these animals through to sexual maturity. This might well repay the effort.

Crepidula

Of the three Woods Hole species of *Crepidula—plana*, *fornicata*, and *convexa—plana* is most commonly used for embryological work. Conklin's studies were made for the most part on the egg of *C. plana.*

The breeding season for *C. fornicata* in the Woods Hole region lasts from early summer to about the middle of August, that of *C. plana* begins somewhat later and lasts longer. The egg-laying season of *C. convexa* covers about the same period as that of *C. plana.* (Conklin, 1897.)

The fertilized eggs in all three species are laid in capsules. This fact guarantees normal eggs. To obtain the eggs one simply removes and punctures the capsules found beneath the snail. All eggs produced by one female are laid at about the same time. Development from egg-laying to the escape of the veligers is very slow, taking about four weeks in the case of *C. fornicata* and somewhat longer in *C. convexa* and *C. plana* (Conklin, loc. cit.). For problems on determinate cleavage this is an excellent egg.

Aplysia

Both at Naples and at Roscoff the large nudibranch, *Aplysia*, is very common. After having been collected, the animals are placed in pairs in large aquarium jars under gently flowing seawater. For the early stages of development, it is necessary to keep the animals under observation, especially once copulation has begun, in order to remove the eggs for study and expier-

ment as soon as they are laid. The egg of *Aplysia* is very hardy and stands up well under laboratory conditions. I have kept eggs developing perfectly for two weeks after they had left the jelly strings (at Roscoff).

Loligo

The squid, *Loligo pealii*, commonly found on the Atlantic coast of America, will breed in laboratory tanks provided they are not overcrowded and the sea-water does not flow too forcefully. By keeping the animals under observation, one can follow copulation and egg-laying as described by Gilman A. Drew (1911). In this fashion one can obtain eggs in various stages of development. Since the females tend to deposit the egg-strings in the same place, it is best to keep the animals in separate pairs.

Eggs removed from the female can be readily fertilized; development in this case is normal. The mechanism of the discharge of the spermatozoa, as worked out by Drew, is an interesting phenomenon worthy of observation.

Cynthia, *Molgula*, *Ciona*

According to Conklin (1905), "the eggs of *Ciona* and of *Molgula* are laid in the early morning, a little before daybreak, while those of *Cynthia* are laid in the late afternoon, a little before sunset." He also states that a large proportion of the eggs of *Cynthia* never develops if fertilized although eggs seem ripe and the spermatozoa active. He found it best, therefore, to use normally laid and fertilized eggs. It is my experience that, although it is best to use normally shed eggs and spermatozoa, ascidian eggs removed from the animals fertilize in high numbers if they be thoroughly washed free of body fluid.

Eggs of *Ciona* taken from the animals fertilize readily. At Naples, at least, eggs and spermatozoa taken from the same individual and mixed give as good development both as to per cent. and as to quality as those obtained by mixing eggs and spermatozoa from different individuals. (Fuchs, 1914; Just, 1934.) Thus my experience with respect to self-fertilization in *Ciona* is similar to Fuchs'. Perhaps the failure of Morgan and of others to obtain self-fertilization in this animal at Woods Hole was due either to the poor condition of the animals which they used or to insufficient numbers of them. I suspect in Morgan's

experiments at least that the failure was due to both, for his published papers clearly give basis to warrant this suspicion. At Naples, *Ciona* is extremely abundant.

Phallusia

Phallusia is also readily obtained at Naples. Its beautiful and transparent egg can be removed from the animal and fertilized with ease. Nevertheless, if one intends to work on stages subsequent to the initial fertilization-reaction, one would best use normally laid eggs.

Amphioxus

For the early stages in development of the egg of *Amphioxus* the worker should collect the animals himself or accompany the collector. At Naples, where *Amphioxus* is abundant, I was taken by the collectors to the collecting ground. There, using a flat rock as laboratory table, I set up my microscope, glassware, etc. As soon as sand containing the animals in large glass dishes was brought me, I removed them to clean sea-water. The ripe animals discharge their sexual products at once. To insure most normal development, it is necessary to place the shedding female into the dish or vessel in which a male had shed, for, as has been pointed out by older investigators, the eggs do not develop normally if they are allowed to lie in sea-water before inseminated. Occasionally, *Amphioxus* shed in the laboratory, as Lwoff (1892) first pointed out, a phenomenon which I observed also in 1929. Shedding by *Amphioxus* in the laboratory has also been observed by Orton at Plymouth, England. (1913–1915.)

Fundulus

Three species of *Fundulus* are used at Woods Hole—*heteroclitus*, *majalus* and *diaphanus*. Newman (1907 and 1909) has described their normal processes of copulation and egg-laying. If pairs of *Fundulus*, *F. heteroclitus* particularly, be isolated, his observations may be readily confirmed. In order to observe copulation and egg-laying by *F. majalus* in captivity, one should place three or four males with one female (Newman, 1909). I have made these observations on both these species but not on *F. diaphanus*. These normally laid eggs are the best to use.

Eggs and sperm can also be obtained by stripping the animals. The stripping should be gently performed by applying pressure on the abdomen toward the anus. The eggs are best fertilized dry, i.e., the eggs and sperm are first mixed and then sea-water is added; this is generally true for teleostan eggs. Personally, I prefer to use normally laid eggs.

Eggs of *Fundulus* are extremely hardy and the fish are easily reared in the laboratory. They are therefore excellent for many problems in experimental embryology. However, they do present to the experimenter one serious obstacle; namely, the chorionic membrane. This, fortunately, at least in the later stages of development, can be removed.

Nicholas and later Armstrong (1928) have used the egg of *Fundulus* with its chorion removed. Practically, the normality of its development is not thereby impaired. Removal of the membrane is a most useful procedure for experimental work. I give Armstrong's method in detail:

He removes the chorionic membrane at the stage of closure of the blastopore under a binocular dissecting microscope with dissecting needles and iridectomy scissors. "In removing the membrane special precautions must be taken to avoid injury to the embryo. The following procedure gave uniformly good results: the point of a sharp dissecting needle was pushed into the membrane and the egg rotated so that the tip of the needle within the membrane could be held against the bottom of the dish at an acute angle. A second needle was then drawn across the under side of the first needle so as to make a slit in the membrane large enough for the introduction of the point of the lower blade of the iridectomy scissors. By this means the membrane was readily removed, without exerting any pressure on the embryo. The naked embryos were kept over night in sea-water, during which time a few embryos, which had been injured in the removal of the membrane died. The mortality was usually 4 to 5 per cent."

Literature

Armstrong, P. B. 1928. The antagonism between acetic acid and the chlorides of sodium, potassium, and calcium as manifested in developing *Fundulus* embryos. **Journ. Gen. Physiol., Vol. 11.**

Cohn, E. J. 1918. Studies in the physiology of spermatozoa. **Biol. Bull., Vol. 34.**

Conklin, E. J. 1897. The embryology of *Crepidula*. **Journ. Morph., Vol. 13.**

———. 1905. The organization and cell-lineage of the ascidian egg. **Acad. Sci. Philadelphia.**

Crozier, W. T. 1918. On indicators in animal tissues. **Journ. Biol. Chem., Vol. 35.**

Drew, G. A. 1911. The sexual activities of the squid. *Loligo pealii*. **Journ. Morph., Vol. 22.**

Field, I. A. 1923. Biology and economic value of the sea-mussel, *Mytilus edulis*. **Bull. Bur. Fish., Washington, Vol. 38.**

Fuchs, H. M. 1914. On the conditions of self-fertilization in *Ciona*. **Arch. Entw. Mech., Bd. 40.**

Gerould, J. H. 1907. The development of *Phascolosoma*. **Zool. Jbcher, Anat. Ont., Bd. 23.**

Just, E. E. 1919. The fertilization reaction in *Echinarachnius parma*. II. The rôle of fertilizin in straight and cross fertilization. **Biol. Bull., Vol. 36.**

———. 1922. Initiation of development in the egg of *Arbacia*. III. The effect of *Arbacia* blood on the fertilization-reaction. **Biol. Bull., Vol. 43.**

———. 1929. Breeding habits of *Nereis dumerilii* at Naples. **Biol. Bull., Vol. 57.**

———. 1934. On rearing *Ciona* to sexual maturity. **Carnegie Year Book, 1934.**

Kostanecki, K. 1904. Cytologische Studien an künstlich parthenogenetisch sich entwickelnden Eiern von *Mactra*. **Arch. mikr. Anat., Bd. 64.**

———. 1911. Über parthenogenetische Entwicklung der Eier von *Mactra* mit vorausgegangener oder unterbliebener Ausstossung der Richtungskörper. **Arch. mikr. Anat., Bd. 78.**

Lillie, F. R. 1915. Studies of fertilization. VII. Analysis of variations in the fertilizing power of sperm suspensions of *Arbacia*. **Biol. Bull., Vol. 28.**

———. 1919. Problems of fertilization. **Chicago, Univ. Chicago Press.**

———, and Just, E. E. 1913. Breeding habits of the heteronereis form of *Nereis limbata* at Woods Hole, Mass. **Biol. Bull., Vol. 24.**

Lwoff, B. 1892. Über einige wichtige Punkte in der Entwicklung des *Amphioxus*. **Biol. Centralbl. 12.**

MATHEWS, A. P. 1901. Artificial parthenogenesis produced by mechanical agitation. **Amer. Journ. Physiol., Vol. VI.**

MEVES, F. 1915. Über den Befruchtungsvorgang bei der Miesmuschel (*Mytilus edulis* L.). **Arch. mikr. Anat., Bd. 87.**

NEWMAN, H. H. 1907. Spawning behavior and sexual dimorphism in *Fundulus heteroclitus* and allied fish. **Biol. Bull., Vol. 12.**

———. 1909. Contact organs in the killifishes of Woods Hole, **Biol. Bull., Vol. 17.**

ORTON, J. H. 1913–1915. Notes on experiments on rearing *Amphioxus*. **Journ. Marine Biol. Assoc., Vol. X. N.S.**

RANZI, S. 1931. Ricerche sulla biologia sessuale degli Annelidi. **Pubbl. Staz. Zool. Napoli, 11.**

SCOTT, J. W. 1906. Morphology of the parthenogenetic development of *Amphitrite*. **Journ. exp. Zool., Vol. 3.**

TREADWELL, A. L. 1902. Notes on the nature of artificial parthenogenesis in the egg of *Podarke obscura*. **Biol. Bull., Vol. 3.**

IV

SOME METHODS FOR PRELIMINARY EXPERIMENTAL MANIPULATIONS

The mere handling of living eggs and spermatozoa in the laboratory may influence their viability, their development, their reactions, and thus the results of experiments made with them. The basic rules given above will serve to guard against sources of error due to improper handling. Where, in addition, an experiment demands preliminary manipulations which, themselves experimental, only precede the experiment to be made, these should be strictly controlled in their effect on the cells; certainly, if they induce abnormalities, these should be of a known and small degree. The truth and importance of this statement are self-evident.

As yet, very few procedures have been worked out thoroughly enough to serve as reliable methods for such preliminary experimental manipulations. In this section, I give as preliminary to experiments a few methods that can be easily controlled in their effect on the cells. These methods are the outcome of scrupulous study through many years; the worker may rely on them.

Methods for Removing the Jelly from the Eggs of Echinids

For some kinds of experimental work the removal of the jelly enclosing echinid eggs is frequently desirable. This is generally accomplished by treating the eggs with HCl in seawater. The jelly may also be removed by shaking. Both methods may be injurious to the eggs.

It has sometimes been stated that the presence of the jelly hulls around echinid ova is necessary for the separation of the vitelline membrane, the normal response to insemination (McClendon, Elder, Gray). Upon this statement theories of the mechanism of memrane-separation have been fabricated.

That the presence of the egg-jelly is not necessary for membrane-separation has been abundantly shown by several workers, notably Harvey, Lillie, Just and Hobson. The fact that shed eggs, which in some instances are devoid of jelly, on insemination separate perfectly normal membranes argues against any necessary rôle of the jelly in the process of membrane-separation. Moreover, frequently through shaking, the eggs lose their jelly but retain their capacity to separate membranes. Centrifuging removes the jelly without impairing the normal response of the eggs to insemination. What is true, therefore, is not that the egg-jelly is the *sine qua non* for this response, but that the methods for its removal may be deleterious to the eggs themselves. This is the case with HCl in sea-water.

HCl in sea-water, employed to remove the jelly from echinid eggs, may be harmful to the eggs either because the concentration of the acid is too great or the washings in sea-water subsequent to the acid treatment are not sufficiently thorough. If the concentration of the acid is too great, it blocks fertilization. According to Clowes and Smith (1923), the acid limit (of CO_2-free sea-water) for a ten minute exposure for eggs of *Arbacia* and *Asterias* is about pH 4.4. That is, at this pH and below, the eggs are irreversibly injured.

If the worker insists on using acid sea-water to remove the egg-jelly he may find the following directions useful:

Treat each of four to six lots of eggs from the same female with 50 cc. of HCl sea-water of pH ranging from 3.5 (eggs exposed for five minutes) to 5.0 (eggs exposed for eight minutes). Wash the eggs thoroughly by carrying them over, with as little of the acid sea-water as possible, to 1000 cc. of normal sea-water in a large flat bottom dish of 3000 cc. capacity. Gently stir the water with a circular motion when the eggs have settled, in order to bring them to the centre of the dish. Pipette them off and place in 1000 cc. of sea-water. Take samples of each lot and inseminate. At least one lot should show as many membranes as the untreated inseminated eggs; if no lot gives this result, the eggs have suffered from the acid treatment. If, however, one or more of the samples show normal membranes following insemination, take additional samples from the original lots and without insemination examine each in turn under the

microscope in order to ascertain whether or not the jelly has been removed. Eggs with jelly do not touch each other, eggs without jelly do. Also, if the eggs be placed in a suspension of Chinese ink, made by grinding up the end of a stick of ink in sea-water, the eggs without jelly are enclosed by particles of ink, whilst eggs with jelly are surrounded by a clear space, the jelly hull.

On the whole, I regard removal of the egg-jelly by means of acid sea-water as unsatisfactory. In order to be sure of success one must use several concentrations because of the individual and seasonal variations of the eggs. Under the most favorable conditions the washing subsequent to acid treatment is laborious and time-consuming if one needs a large number of eggs.

Shaking will remove the egg jelly, but in my experience is uncertain when the eggs are at their seasonal optimum. Toward the end of the breeding season the jelly is more easily removed; indeed, eggs then frequently lose their jelly on standing in normal sea-water without any treatment. Nor can I recommend the KCN method of Vlès.

I find that the simplest and most effective method of removing the jelly from eggs of echinids is to put them through bolting silk. In this way the eggs are in no wise impaired, as can be demonstrated by the fact that their membrane-separation is of the same rate, quality, and per cent. as that of eggs from the same female which possess jelly hulls. One merely pours the eggs on to the wetted bolting silk stretched over a finger bowl containing sea-water. There is only one precaution: *one must not use pressure*—e.g., by pouring eggs from a height greater than three or four centimeters. Eggs examined under the microscope in a suspension of Chinese ink in sea-water are found free of jelly. If some eggs still possess jelly they are put through the bolting silk again. I have used this method for several years now. After rather tedious comparisons with the other methods named, I can safely say that it is the best.

Methods for Removing the Vitelline Membrane from Inseminated Echinid Eggs

A great deal of experimental work has been done on echinid ova whose vitelline membranes have been removed after their

separation as the result of insemination or of treatment with an organic acid. The membranes are most readily removed immediately after separation. This removal is generally accomplished by shaking. But there is much evidence to indicate that at this time the eggs are very susceptible to shaking. Boveri and others used shaking for the specific purpose of modifying the development of echinid eggs. I should say that any interpretation concerning normal development based on the study of eggs whose membranes have been removed by shaking the eggs immediately after membrane-separation is without value. Shaking at this time however is an excellent experimental method for the modification of the normal developmental process.

The membranes can, however, be removed without the slightest injury to the eggs. For this purpose again I use bolting silk. The method is as follows:

I use eggs from one female known by previous trial inseminations to be of optimum fertilization capacity, as revealed by the speed and quality of the ectoplasmic reactions induced by insemination. About two minutes after insemination when the membranes are equidistant from the egg surface at all points—i.e., when the perivitelline space is of equal width throughout—the egg suspension is very gently poured on to the wetted bolting silk. As the eggs pass through the mesh, they lose their membranes. Practically 100 per cent of the eggs will thus be freed of their membranes. *Eggs without membranes should never be crowded; they should lie in one layer well spaced in a large volume of sea-water.*

Methods for Inducing Membrane-Separation in Unfertilized Echinid Eggs

In 1893 Herbst repeated and confirmed the observation of the Hertwig brothers that chloroform induces membrane-separation in the sea-urchin egg. He also found that benzol, toluene, xylene, creosote and clove-oil have the same effect.

Schücking found that eggs exposed to distilled water separate membranes. This observation has been confirmed by Glaser and by myself. Hypertonic sea-water will also induce membrane-separation, I find, in several species of echinid eggs. The most popular method for bringing about the separation of the

membranes is that with butyric acid as first used by Loeb on the California sea-urchin.

Which of these methods the worker will use depends upon the objective of his experiment. Certainly, he will choose the most reliable of them. In my experience, the methods employed by the Hertwigs and Herbst are the least valuable. The three remaining have value depending upon the purpose of the experiment. If the aim is to obtain membrane-separation with the least possible degree of induction of subsequent development of the eggs, distilled water, butyric acid or other straight chained fatty acids should be used because with these the eggs never cleave; they only reach the monaster stage. The hypertonic sea-water of strength sufficient to induce membrane-separation will, if allowed to act long enough, induce not only membrane-separation, but cleavage and subsequent farther development, closely simulating the normal.

In either distilled water or hypertonic sea-water the eggs separate their membranes. With butyric acid-sea-water, on the other hand, membrane-separation takes place only after removal of the eggs to normal sea-water. This difference in action of the means the worker must bear in mind in order properly to interpret whatever results he obtains on the basis of such experimentally induced membrane-separation.

To bring about membrane-separation by means of distilled water my procedure is as follows:

Select eggs from a single female which by trial insemination give perfect membrane-separation. Take a thick drop of eggs in a Syracuse watch-glass mounted under low power of the microscope and flood with distilled water. (It is best to use distilled water which the worker himself has re-distilled in a quartz-still; if such quartz distilled water is not available, use tap water.) Now with the stop watch note the time when the membranes are fully separated from the eggs. This observation should be repeated several times. One learns that the time to complete membrane-separation will vary depending upon the species of echinid. Eggs of *Arbacia* in optimum physiological condition separate membranes in distilled water in about 15 seconds. The moment that the membranes are fully off the eggs, they should be brought into a large volume of sea-water.

For eggs in 10 cc. of distilled water, 250 cc. of normal sea-water suffices.

To induce membrane-separation in hypertonic sea-water, the method depends upon the degree of hypertonicity as well as upon the species of egg. With this method my procedure is as follows:

Make up 3 solutions of 2½*M* NaCl or KCl plus sea-water in the following proportions: 20, 22 and 24 parts of the salt plus 80, 78 and 76 parts respectively of sea-water. Flood with the hypertonic sea-water a drop of thick egg-suspension in a Syracuse watch-glass mounted under low power of the microscope. The moment that the eggs separate their membranes, put them into dishes containing at least 250 cc. of sea-water. After several trials with each solution, use that which gives membrane-separation in the shortest time. The rate of membrane-separation will vary somewhat on eggs of the same species even when these, as learned previously by trial insemination, are in the best physiological condition.

I find that pure 2½*M* NaCl or KCl will bring about membrane-separation often in less than 1 minute. But with this solution, the eggs are induced to complete development. Thus, this is a method not only for membrane-separation, but also for experimental parthenogenesis.

The butyric acid method employed by Loeb (1913 and earlier) has been extensively used by others. Here again the method varies depending upon the species of egg. The important point is to avoid over-exposure to the butyric acid-sea-water, for eggs over-exposed do not separate full membranes; they are injured without loss of capacity for fertilization. One should also avoid using too great concentration of the acid. The method as modified by Heilbrunn and used subsequently by F. R. Lillie, C. R. Moore and myself for the egg of *Arbacia* is applicable, I find, to eggs of other echinids. My use of this method follows:

Have prepared a cylinder containing 50 cc. of sea-water plus 2 cc. $\frac{1}{10}$ normal butyric acid. Shake the cylinder vigorously. Add the butyric acid-sea-water to eggs in a flat-bottom glass vessel. At 10 second intervals remove about 1 cc. of the egg-suspension to 250 cc. of sea-water. In this wise establish a series consisting of 10 to 12 numbers. Examine samples from each dish and estimate the percentage of eggs with fully sepa-

rated membranes in order to determine which exposure gives the highest per cent. of separated membranes. Repeat the experiment removing the eggs now at 5 second intervals beginning 10 seconds before the stage that proved to be best in the first series of experiments, and continue 10 seconds beyond. Inspect the eggs again and choose that exposure which is optimum.

Methods for Inducing Membrane-separation in Unfertilized Eggs of the Genus Asterias

Methods for calling forth membrane-separation in eggs of *Asterias glacialis*, *A. vulgaris* and *A. forbesii* comprise the use of carbon dioxide, high temperature and butyric acid. The first we owe to Delage (1902*a*, *b* and *c*); R. S. Lillie (1908, 1916) especially has used the second and third. With any one of these methods, it is essential to use eggs whose germinal vesicles have disrupted.

Membrane-separation may be induced by means of carbon dioxide either by exposing the eggs to sea-water charged with it, or by crowding the eggs in sea-water. For the former a convenient apparatus is the ordinary syphon used for charging water, the so-called sparklet bottle, as employed by Delage. One fills the bottle to the red mark with clean sea-water and charges it with the gas from the steel cartridge. The eggs are flooded with the CO_2 charged sea-water and removed at 10 second intervals. In this wise, the optimum length of exposure is learned. Another set of eggs is treated with the charged sea-water and the eggs removed at intervals around the optimum exposure as previously determined in order more exactly to fix the optimum exposure. This method is nicer than that of crowding the eggs in a small quantity of sea-water.

For the method of using butyric acid in sea-water for calling forth membrane-separation in the egg of the starfish as well as for that by means of heat, the worker may consult the original papers by R. S. Lillie.

Methods for Inducing Experimental Parthenogenesis

The worker who wishes extensive treatment of the subject of experimental parthenogenesis has available reviews, as those by

Delage, Herbst and Loeb. Of these that by Delage is best (1910). It is my purpose to detail only those methods that I know first hand. Also, I make a distinction that the authors named above did not make, namely, that between methods for inducing initial changes in the eggs without cleavage and subsequent development and those which give development to a swimming form. I think it is well to restrict the meaning of the term, experimental parthenogenesis, so that it does not include those effects upon eggs which do not lead to development. The work on experimental parthenogenesis has suffered greatly because of failure to make this distinction.

The best means for calling forth development of marine eggs to a swimming stage is hypertonic sea-water. Its effect varies depending upon the species of egg, and, for a named species, upon the degree of hypertonicity. With respect to the former, it may induce wholly abnormal swimming forms, as for example, on eggs of worms which develop without cleavage; as to the latter, it may on the eggs of echinids produce swimming forms without having initiated complete ectoplasmic change. Another effective means is increase in temperature. A third, acid in sea-water. Hypotonic sea-water succeeds in some cases, but in my judgment is a rather poor means. The same may be said of radiations and some other means, whose effects have been investigated. Another method consists in combining two means, as the butyric acid hypertonic sea-water method which really is a combination of three means, since it is only successful when the eggs are placed in sea-water between exposure to each agent.

Hypertonic sea-water

Hypertonic sea-water brings about development to the swimming stage without cleavage, so-called differentiation without cleavage, in eggs of many worms especially. Unless the worker desires to make experiments on this type of development, he should not use hypertonic sea-water on these eggs, as for example, those of *Nereis*, *Chaetopterus*, *Amphitrite*, etc. If, however, he has in mind to investigate differentiation without cleavage, he should consult the literature on this subject for the methods used.

Acting on echinid eggs, weak hypertonic sea-water made up by adding 8 cc. of 2.5*M* NaCl to 50 cc. of sea-water, as originally used by Loeb, calls forth development without well separated membranes, with poor cleavage and with swimming forms that do not rise to the surface of the water. Again, unless the worker intends frankly to study this phenomenon, he would do well not to use this method. This weak hypertonic sea-water is used best in combination with butyric acid, as given beyond.

Hypertonic sea-water calls forth development closely simulating the normal in several species of eggs. Among these are eggs of molluscs, worms, the starfish and echinids. Since the echinid egg is the one most extensively employed in the study of experimental parthenogenesis, I give the methods which I have found to be the best for inducing full development in it (1922).

From a single female take a thick drop of eggs known from previous trial inseminations to be in optimum physiological condition. Flood with 10 cc. of 2½*M* NaCl or KCl in a watch-glass mounted under low power of the microscope. Note exactly with the stop watch the time to complete membrane-separation. In the majority of cases one will find that 40 to 60 seconds exposure is sufficient. Remove eggs to 250 cc. of normal sea-water. With this method, the eggs should cleave and 90 per cent. should develop into top-swimming plutei.

Hypertonic sea-water may also be used by making up sea-water plus 2½*M* NaCl or KCl in the following proportions: the salt solution in a series beginning with 95 parts and decreasing by grades of 5 parts to 25 parts plus the sea-water beginning with 5 parts and increasing in the same way to 75 parts respectively. In these solutions, the duration of the effective exposure increases with the amount of sea-water employed. All of these concentrations except the last named require quick manipulation on the part of the observer. Hence, he may prefer to use the following:

To a drop of eggs in optimum physiological condition is added hypertonic sea-water made up of 80, 78 or 76 parts of sea-water plus 20, 22 or 24 parts respectively of 2½*M* NaCl or KCl. Under the low power of the microscope the time to membrane-separation is noted and the eggs are removed there-

after at 30 second intervals to dishes containing 250 cc. of sea-water. In this wise, the optimum exposure for development to the pluteus stage is determined.

Increase in temperature

Increase in temperature has been used for the experimental initiation of development, especially by R. S. Lillie on the starfish egg. Allyn has used it on the egg of *Chaetopterus*. According to her, this is the only means which gives development with cleavage in this egg. I have used elevation of temperature on the egg of *Nereis* and have found that it gives a development (with cleavage) which is scarcely to be distinguished from the normal. For the egg of *Nereis*, increase in temperature may be employed in two ways.

1. On the evening of capture, or at latest the morning after, females having been carefully washed in gently running sea-water are rapidly dried on soft filter paper and placed in tubes containing sea-water at temperatures of 30, 31, 32 and 33°C. The females should shed the eggs at once. The eggs extrude the jelly immediately thereafter. At 5 minute intervals, pipette a drop of eggs to finger bowls containing sea-water at room temperature (18 to 20°C.). The effective exposure gives more than 90 per cent. trochophores.

In this method, it is important that the eggs come into the warm sea-water without having been in sea-water of lower temperature. If, for example, the eggs are washed in sea-water by removing them from the female to sea-water at room temperature, they will not respond to warm sea-water, although they have suffered no diminution in fertilization-capacity. If the eggs are shed during the process of drying the female, they may be successfully exposed to the warm sea-water. However, it is best not to cut the female in order to obtain the eggs, for thus blood introduced with the eggs into the warm sea-water lowers the quality and per cent. of the response. (Just, 1915.)

2. I have used also the method of exposing the eggs to higher temperature for about 60 seconds. In this method, the eggs may be removed from sea-water at room temperature to sea-water warmed to 40°C. But the response of the eggs is not as good as in the case of eggs shed directly into the warm sea-water.

Acid in sea-water

Of the various acids, mineral and organic, employed as means for calling forth development to at least the larval stage, butyric acid is most used. The work on *Thalassema* ought be noted, but I can not comment upon it because of lack of experience with this egg. I limit myself to the egg of *Asterias*. Carbon dioxide is as effective as butyric acid. I suggest therefore that the worker who has in mind an investigation on the effect of acid in calling forth complete development, use the carbon dioxide method as prescribed by Delage for the egg of *Asterias glacialis*, or the butyric acid method as employed by R. S. Lillie on the egg of *A. forbesii* or *A. vulgaris*.

Double treatment

By double treatment I mean the effect of two different means, each of which calls forth a different response from the egg. Thus I do not include the action of two treatments with butyric acid on the egg of the starfish or that of butyric acid followed by heat, as used by R. S. Lillie. True double treatment is that as used by Delage in his tannin-ammonia method for the echinid egg, or that employed by Loeb. The Loeb method is the more common. This is as follows:

Uninseminated eggs are placed in 50 cc. of sea-water plus 2 cc. of $N/10$ butyric acid for one and one-half to three minutes, and removed to normal sea-water. Ten to fifteen minutes later the eggs are removed from sea-water to 50 cc. of sea-water plus 8 cc. of 2.5M NaCl. From this hypertonic sea-water they are returned to normal sea-water at intervals between seventeen and one-half and twenty-five minutes.

Heilbrunn (1915) modified Loeb's method by taking 2.8 cc. of $N/10$ butyric acid, instead of 2 cc., added to 50 cc. of sea-water, which was allowed to act for 30 seconds. With this method, he obtained 90 per cent. separated membranes in the egg of *Arbacia* instead of the gelatinous films described by Loeb.

For the egg of *Echinarachnius* (Just, 1919), I use 2 cc. $N/10$ butyric acid plus 50 cc. of sea-water acting for 35 seconds. The subsequent residence in sea-water and treatment with the hypertonic sea-water are as for the egg of *Arbacia*.

On the basis of my experience with the eggs of these two forms and with several species of echinids in European waters, I suggest as general rule of procedure the following:

(1) Take always unfertilized eggs known by previous trial inseminations of samples of them to be in optimum fertilizable condition. (2) Expose them to 2 cc. N $\frac{1}{10}$ normal butyric acid added to 50 cc. of sea-water and thoroughly dissolved by vigorous shaking. (3) At ten second intervals remove 1 cc. of the eggs to 250 cc. of sea-water, establishing thus a series of 10 to 12 numbers in order to determine that exposure which gives the highest per cent. of membrane-separation. (4) Expose to the butyric acid sea-water another lot of eggs from the same female for that length of time found to be the optimum, removing the eggs all to 1000 cc. of sea-water. (5) Remove at intervals of five minutes 4 lots of the eggs from sea-water each to 50 cc. of sea-water plus 8 cc. of 2.5M NaCl. (6) At intervals of five minutes during twenty-five minutes remove samples of the eggs from the hypertonic sea-water to 1000 cc. of normal sea-water. By (5) and (6) one establishes (a) the optimum residence of the eggs in sea-water and (b) their optimum exposure to hypertonic sea-water for the production of top-swimming plutei.

Literature

Clowes, G. H. A. and Smith, H. W. 1923. The influence of hydrogen ion concentration on the fertilization and growth of certain marine eggs. **Amer. Journ. Physiol., Vol. 64.**

Delage, Y. 1902*a*. L'acide carbonique comme agent de choix de la parthénogénèse expérimentale. **C. R. Acad. Sci. CXXXV.**

———. 1902*b*. Sur le mode d'action de l'acide carbonique dans la parthénogénèse expérimentale. **C. R. Acad. Sci. CXXXV.**

———. 1902*c*. Nouvelles recherches sur la parthénogénèse expérimentale chez *Asterias glacialis*. **Arch. Zool. Exp. Sér.3. X.**

———. 1910. La parthénogénèse expérimentale. **Verh. VIII. Internat. Zool. Kongress., Graz.**

Heilbrunn, L. V. 1915. Studies in artificial parthenogenesis. II. Physical changes in the egg of *Arbacia*. **Biol. Bull., Vol. 29.**

Just, E. E. 1915. Initiation of development in *Nereis*. **Biol. Bull., Vol. 28.**

———. 1919. The fertilization reaction in *Echinarachnius parma*. III. The nature of the activation of the egg by butyric acid. **Biol. Bull., Vol. 36.**

———. 1922. Initiation of development in the egg of *Arbacia*. I. Effect of hypertonic sea-water in producing membrane separation, cleavage and top-swimming plutei. **Biol. Bull., Vol. 43.**

Lillie, R. S. 1908. Momentary elevation of temperature as a means of producing artificial parthenogenesis in starfish eggs and the conditions of its action. **Journ. Exp. Zool., Vol. 5.**

———. 1916. Mass action in the activation of unfertilized starfish eggs by butyric acid. **Journ. Biol. Chem., Vol. 24.**

Loeb, J. 1913. Artificial parthenogenesis and fertilization. **Univ. Chicago Press, Chicago.**

V

METHODS OF FIXATION

The worker who plans extensive study on fixed eggs will consult any one of the many books on microscopy and on microtomy for the methods of fixation and staining. There are in addition various journals for technique in microscopy. Finally, almost every text-book of histology devotes some pages to methods for preparing tissues for microscopic study. Thus, there is no good reason for my giving an exhaustive treatment of the subject. As in the foregoing pages, I here confine myself to some methods that have proved valuable to me in the course of thirty years of experience with animal eggs.

The Value of Fixed Eggs

Undoubtedly there are shortcomings to the use of fixed cells. Certainly, the experimental embryologist, as any investigator interested in protoplasmic behavior in its living and completely viable condition, needs to be careful in drawing conclusions concerning processes in the living organism on the basis of too great, to say nothing of exclusive, emphasis on the fixed object. The fixed cell, as a dead cell, can obviously give us only a picture of what was once alive. On the other hand, the permanence of structure in the fixed cell argues very strongly that the components have place in the living. Thus the cellular structures which have been most carefully worked out are those which withstand to the greatest degree changes brought about through fixation, as, for example, the nucleus and its components, the chromosomes. These, the most rigidly static structures in the cell, suffer least in fixation. The study of fixed cells does not deserve the wholesale objection often raised against it. If the experimental embryologist appreciates the limitations of fixation, he can use it to great advantage. The study of the fixed egg is a valuable aid, supplementing observation and experiment on the living.

The investigator by such study can correlate the changes observed in the living system more exactly by comparing the changed states in the fixed. The rapidity with which many events in a developing egg take place is such that one can not easily follow them with assurance in the living. If, however, the worker has repeated his observations on the living, he can by use of fixed stages come to surer knowledge as to the sequence of events and to the predominating factor both of which may escape him in direct observation on the living egg. Indeed, in some cases, as for example, changes in large or in opaque eggs, he can follow these changes exactly only in fixed preparations. A great deal of work in experimental embryology and even that in physiology could be much improved by correlating the changes examined in the living state with those revealed by the fixed.

The Choice of the Fixative

The choice of the fixative employed is of paramount importance. Since the fixed egg is a dead egg, the method of bringing about the death-change is a primary consideration: the aim is to secure fixation which will distort the structural organization of the egg in the least possible degree. Thus, the fixative should act very rapidly; it should penetrate quickly and yet without violence; it should fix evenly, without disrupting the relation of the various regions in the cell. This applies to any fixing agent used, regardless of the cell component studied.

A second consideration concerning fixation involves the cellular component under study. If we are interested in nucleus and chromosomes, the fixative employed is that which most faithfully preserves their structure. Although these are perhaps the cell structures that can be fixed most easily, not all fixing solutions that are capable of giving good nuclear and chromosomal fixation have the same excellence.

If one has interest in the study of cytoplasmic inclusions, as mitochondria, yolk-spheres or oil-drops, one should use a fixative that preserves the inclusions in a condition that resembles as closely as possible that found in the living. Inclusions, especially mitochondria, often demand special methods. Cell pigments also require special treatment.

Similarly, the fixative employed for the cytoplasm, the menstruum in which the cytoplasmic inclusions lie, should be one which gives a picture that simulates the cytoplasmic structure of the living cell. Such a fixative should be as perfect for the cytoplasm holding the inclusions as for the cytoplasm that has been freed of inclusions. Depending upon the object studied, the fixative may or may not be the same as that which fixes the nucleus or the inclusions of the given cell.

At the surface of an egg the cytoplasm has structure which differs from that more centrally located. This, the ectoplasm, so highly mobile, registers changes in the environment and reveals differences in structure that run with the activity of the cell. Its structure as seen in the living egg is faithfully reproduced only with the aid of certain fixatives.

There is no one fixing solution that will serve for all structures found in an egg. This statement is true for animal cells in general. Moreover, that a named fixing solution gives excellent results when used on one species of egg, does not mean that it will succeed on another. *My first suggestion, therefore, is that the worker should try out several fixing solutions in order to ascertain that which is best for his purpose, always of course comparing the fixed egg with the living.*

A very simple observation that I have made may be mentioned. Let us take a few developing eggs mounted in a hollow microscope slide under low power of the microscope and run on to them a fixing solution, noting the time in seconds when the eggs are killed without any change in form. If, for example, we take drops of an egg suspension at the time when the first cleavage furrow is appearing and try out fifteen to twenty fixing solutions, we note that in some cases the blastomeres change in shape before they are killed; in others, the blastomeres are killed in exactly that stage in which they were when subjected to the solution. For the echinid egg I have found that some descriptions of cell division based on fixed eggs are at fault because of the use of a fixing solution which alters the shape of the egg.

Although, in general, the volume of the fixing solution plays a part in determining results obtained, this is not the case in the foregoing observation, because the same result is obtained

regardless of the ratio of volume of eggs to volume of fixing solution. In other words, those fixing solutions that alter the shape of an egg very greatly will do so regardless of the amount of solution used. One should nevertheless be at pains to use large volumes of fixing solutions.

Fixatives Most Commonly Used on Eggs

Fixatives most commonly employed for studies on eggs are those containing mercury bichloride, picric acid, formol, chromic acid, or osmic acid. But these are scarcely ever used alone. Generally, fixing solutions contain acetic acid, especially for the fixation of nuclei and chromosomes.

Mercury bichloride

Of the various solutions containing mercury bichloride, Gilson's reagent is in my judgment one of the best, although Lang's is good also. The formula for Gilson is as follows:

Nitric acid (of about 80 per cent)	15 cc.
Glacial acetic acid	4 cc.
Mercury bichloride	20 gr.
60 per cent. alcohol	100 cc.
Distilled water	880 cc.

After fixation with Gilson the eggs are brought directly into 70 per cent alcohol containing iodine to dissolve the fine crystals of the sublimate. The eggs may be kept in 70 per cent alcohol until they are to be imbedded if sections are to be made or until they are to be prepared for study *in toto*. Eggs kept in Gilson for weeks do not suffer in any appreciable degree.

Lang's is 95 parts of a saturated aqueous solution of mercury bichloride plus 5 parts glacial acetic acid.

After fixation with Lang, the eggs should be washed in water, then run up in successive grades of alcohol to 70 per cent alcohol containing iodine, where they are kept until they are to be mounted or imbedded.

Picric acid

The older fixing solutions containing picric acid, as Kleinenberg's, Boveri's, etc., are still often employed. On the whole,

I think, these, if they have value, are for *in toto* mounts only. The addition of chromic acid to fixing reagents containing picric acid I have never found as valuable for marine eggs as workers have found it to be for tissue cells generally.

Of the many fixing reagents containing picric acid by far the best is Bouin's. The formula for this is as follows: 75 parts saturated aqueous solution of picric acid plus 25 parts formol plus 5 parts glacial acetic acid.

After fixation with Bouin's, the eggs are removed to 80 per cent. alcohol containing a few crystals of lithium carbonate with repeated changes until no more yellow color tinges the alcohol.

Chromic acid

Fixatives containing chromic acid are Zenker's, Tellyesniczky, Flemming, etc. Zenker's I do not recommend for marine eggs. Tellyesniczky is good in some cases. Its formula is as follows:

Potassium bichromate	3 grms.
Glacial acetic acid	5 cc.
Water	100 cc.

Since 1910 I have used a modification of Tellyesniczky, which consists of the addition of 10 cc. of formol to 90 cc. of Tellyesniczky's solution. In this case, the formol is added immediately before the fixative is used.

Osmic acid

Many competent cytologists do not employ osmic acid because of some very serious drawbacks to its use. Osmic acid in fixing solutions is notoriously poor in power of penetration. It is often a serious hindrance to staining. Where it is allowed to act too long, it produces what is spoken of as "over-osmification"; such sections give a blackened and muddy appearance. In my experience, these obstacles may be overcome.

In the first place, one may use osmic acid alone or together with other reagents with assurance of perfect results if one takes objects of sufficiently small size. Thus it need not fail when used on the minute eggs of marine animals. Blocks of tissue

cells are successfully fixed by such a fixing solution only if the object is of a much smaller size than that used in fixation by the more generally employed agents. Gut cells of mammals, kidney cells of various vertebrates may be most beautifully fixed by solutions containing osmic acid, provided the cells are removed in strips or isolated into their constituent layers: mucosa cells of the gut and a small length of kidney tubules separated from contiguous layers give perfect fixation, whereas a block of cells equal in bulk to the strip does not. One factor in fixation with solutions containing osmic acid is the presence of fat or yolk. If the tissues to be studied are rich in these, the difficulty of fixation is increased. But even this is not a serious obstacle, if one takes small enough pieces. This fact is shown in a simple way. Of an emulsification of acid and olive oil with egg yolk as emulsifying agent, mayonnaise, I have fixed individual drops of successive diminution in size. The osmic acid blackens each drop; but in the case of the larger drops it does not penetrate even after weeks; cutting into these revealed the interior as yellow as the original mayonnaise from which the drops came. The smaller drops, on the other hand, those ranging in size from 40 to 200 microns in diameter, are blackened throughout in less than an hour. Such droplets may be treated as though they were eggs, that is, sectioned and stained.

The difficulty of over-osmification, where this obtains, can be overcome by not allowing the object to remain too long in the fixing solution. In my experience, however, the problem of over-osmification is often caused by a too great amount of the acid in the solution as employed. If the proper amount of osmic acid is used, cells do not show over-osmification even if they remain in the fixative for weeks. I should point out that often tubes contain more osmic acid than the weight named on the label. I suggest that one use 4, 3, 2, and 1 cc. of this acid made up in 2 per cent. solution. It is perhaps also true that workers experience difficulty with the use of osmic acid because they do not wash it out sufficiently.

Against these shortcomings of osmic acid stands a compensation: the ease in handling eggs having been fixed by it. It is a great nuisance to collect eggs which have been fixed in solutions

of formalin, mercury bichloride, picric acid, or in fixatives containing these chemicals. One loses eggs on transferring them, in washing and in dehydration. Any solution which contains osmic acid insures the worker that he may retain every single egg fixed. The osmic acid so increases the specific gravity of the eggs that they settle very rapidly in all of the stages of washing, dehydration and clearing. In paraffin, eggs having been fixed in most solutions tend to float on the surface. They scatter widely and settle slowly, sometimes not at all. One never has this trouble in imbedding eggs having been fixed in solutions containing osmic acid.

When osmic acid is used in combination with chromic acid, another good feature obtains. Many marine eggs are enclosed in jelly. This jelly is often a source of great annoyance; in some fixing solutions it never dissolves. The presence of chromic acid insures the complete but harmless dissolution of the jelly. Also, whereas osmic acid may be unfavorable for subsequent staining, chromic acid for many stains is favorable. In some cases staining capacity may be increased by treating the eggs with potassium bichromate. I have also used the old method of treating eggs, fixed in solutions containing osmic acid, with picric acid. By and large, however, I think that the use of solutions containing osmic acid in the way that I shall suggest, obviates the necessity of any subsequent treatment with other reagents.

Osmic acid alone I seldom use except for fixing rapidly occurring changes in the ectoplasm of eggs, as those due to sperm penetration, for example, that I wish to study in the fixed condition for the purpose of orienting experiments or for the purpose of comparing its action with that of solutions of it in combination with other reagents. For the most part I employ osmic acid in a solution of chromic acid or in one of potassium bichromate.

Flemming's solution and modifications

Osmic and chromic acids with the addition of glacial acetic acid make up several fixing solutions, Flemming's and modifications thereof. Thus, are available: Weak Flemming, Strong Flemming, and modifications by Benda and by Meves.

Table

WEAK		STRONG	
Flemming		Flemming	
1 per cent chromic acid	25 cc.	1 per cent chromic acid	15 cc.
1 per cent osmic acid	10 cc.	2 per cent osmic acid	4 cc.
1 per cent glacial acetic acid	10 cc.	Glacial acetic acid	1 cc
Water	55 cc.		

Benda

1 per cent chromic acid	15 cc.
2 per cent osmic acid	3 to 4 cc.
Glacial acetic acid	3 drops

Meves

0.5 per cent chromic acid	15 cc.
2 per cent osmic acid	3 to 4 cc.
Glacial acetic acid	3 drops

The Strong Flemming I find superior to the Weak Flemming. In turn, the Meves, as I use it, is better than the Strong Flemming—a statement that I make on the basis of experience in fixing animal cells, other than eggs, from Protozoa to mammals, including normal and pathological human tissues.

Author's method of using Meves' solution

Although, as I have said before, there is no all-purpose fixing solution, the Meves solution as I modify it comes nearest to being such. (Just, 1933.) Where it fails, Altmann's potassium bichromate solution succeeds when I vary the proportions of the ingredients until I get just the right combination for the cell under study. I would greatly urge cytologists to overcome the prejudice against, or fear of, the use of these osmic acid containing fixatives.

My modification of Meves is very simple: it consists merely of varying the amount of the osmic acid used for each species of egg or cell. Instead of using only 4 cc. of the 2 per cent. osmic acid solution, I use, in addition, 3.5, 3, 2.5, 2, 1.5, and 1 cc. before I decide which is best for the object under study. I take finally that modified solution of the Meves that contains the least amount of osmic acid that blackens the cells. Others, botanists and zoologists, who have used this simple method since it was published (1930) have told me that they find it eminently superior to the Flemming or to the Meves.

Meves' solution as shown in the table given above contains half the amount of chromic acid and one fifth to one tenth less of glacial acetic acid than the Flemming. That these are important differences, can be shown by fixing cells of kidney, pancreas, small intestine of the rabbit, guinea pig and rat; eggs of various invertebrates, including those of cephalopods and of crustacea and eggs of bony fishes and blastoderms of chicks; testis cells of *Protenor belfragi*, *Anasa tristis* (Hemiptera), *Gryllus*, *Romalea* and other Orthoptera as well as *Paramaecia* and other Protozoa. In every case it has been my experience that the Meves with less chromic acid and acetic acid and with the lesser amount of osmic acid gives superior fixation.

With Meves as I use it nuclei and chromosomes are beautifully fixed. The configuration of the mitotic figure observed in the living egg is preserved almost without change. In addition, mitochondria are so well fixed that one need not employ special stains to reveal them; they show up perfectly with haematoxylin, as we shall see later. The yolk-spheres are preserved intact and the oil is not dissolved out. These points one can determine for oneself by comparing the structure of these bodies in a fixed preparation with their structure in a living egg, like that of a nereid worm, in which the oil and yolk are clearly revealed in the living state. In Flemming the oil is dissolved and the yolk is badly fixed; the mitochondria are not preserved. With reference to the oil-drops, a centrifuged echinid egg makes a striking example, for after the modified Meves fixation the oil-drops constituting the gray cap at one pole of the living egg can actually be counted in the sectioned egg.

The cytoplasm of marine eggs with which I am familiar is by and large well fixed by any one of a number of fixing solutions. However, a fixative, as is the case with most, that dissolves out the oil and distorts the yolk-spheres even when these are not dissolved, is apt to cause some change in the cytoplasm. Even in centrifuged eggs, in which the cytoplasm (ground-substance) is separated as a clear zone, the dissolution of the cytoplasmic inclusions undoubtedly has some effect. It is, therefore, better for investigation on the cytoplasm itself to employ a fixative that does not have these deleterious effects on the inclusions. Also, it is reasonable to suppose that a fixative that preserves all

inclusions within the cell-membrane in a fashion which most closely resembles the living state, is to be preferred to one that does not. My modification of Meves meets these demands.

Only in one respect do eggs fixed with this modified Meves fall short of complete resemblance to the living: this fixative does not preserve the delicate ectoplasmic structure. For this, therefore, one must use some other fixing agent. Of these, the best in my judgment is Altmann's. In some cases, formalin, provided it is of the best grade procurable and preferably free from formic acid, is a valuable fixative. In others, corrosive sublimate may be used.

Altmann fixation

Altmann's fixative is made up as follows:

2 per cent osmic acid	50 cc.
5 per cent potassium bichromate	50 cc.

Depending upon the egg under study, I reduce the amount of osmic acid sometimes to 25 cc. Also, I frequently take 3 per cent potassium bichromate. Again, I use often the formula employed by Mathews (1901) in his study on the amphibian pancreas, a fixation which I find most excellent.

Washing

Since Meves' and Altmann's solutions contain chromic acid and osmic acid, objects fixed in them must be washed in water. This is in accordance with the general rule that cells fixed in solutions containing salts of heavy metals should be washed in water. (1) Where the object is large enough, so that the danger of its being lost during washing is small, it should be washed in running water for 12 to 24 hours depending upon its size. (2) Eggs may be washed by placing them in a large dish of 2 to 3 liters capacity into which tap water drips very gently. (3) Or they may be placed in glass tubes covered with fine bolting silk and gently inverted so that the eggs come to lie on the silk; this silk-covered end of the tube is placed on an inclined trough, on to which tap water is run. The difficulty of washing by either method (2) or (3) 30 to 40 sets of eggs from each of 10 or more experiments which would represent a season's work at the sea-

side is obvious. I have made very careful comparisons between eggs of *Nereis limbata*, *Chaetopterus*, *Echinarachnius* and of *Arbacia* washed during 24 hours and those washed for one hour by the method which I shall now give and have been unable to detect any difference as the result of the prolonged washing. Eggs washed for an hour are stained as beautifully as those washed in running water for 24 hours.

The eggs are washed for one hour by the simple expedient of changing the water as often as the eggs settle, gently inverting the vial after each change; the eggs settle quickly and when this has taken place, the water is withdrawn and replaced.

Dehydration

Following washing the eggs are dehydrated by being changed to grades of alcohol of increasing strength. In each grade the eggs remain for 1 hour. The grades used are 35, 50, 70 and 80 per cent. Some workers omit the lower grades; this I think is a bad practice. On the other hand, some begin the dehydration with 15 per cent alcohol, going from it to 25, then to 35 and further as given above. I have been unable to detect any difference between eggs the dehydration of which was begun at 15 and those whose dehydration was begun at 35 per cent alcohol.

In 80 per cent alcohol the eggs may remain until the worker is ready to imbed them. I would very strongly urge that they be not kept in the 80 per cent alcohol any longer than necessary. I always endeavor to imbed my eggs as quickly as possible after they have been fixed. The maximum time that I allow them to remain in 80 per cent alcohol is two weeks, although in exceptional cases I have not imbedded the eggs until return from the sea-side to an inland laboratory, about four weeks later. In addition to the practical advantage of transporting eggs in paraffin blocks rather than in alcohol is one far more important yet to the experimental embryologist: by rapidly completing the procedure of fixation and staining, he can learn enough from the sections to orient him in making additional experiments during the current season.

From 80 per cent. alcohol the eggs are carried through final stages preparatory to imbedding in paraffin. These stages will vary depending upon the method to be used in the staining of the

sectioned egg. Here I take up the simplest and the most commonly employed method for these preparations, leaving for later consideration my method for preparing special staining.

Final stages of dehydration

The final stages of dehydration are important because it is in the alcoholic solutions above 80 per cent that cells suffer most, especially from shrinkage. The changes to successively stronger solutions should therefore be carried out in the minimum time that insures dehydration. Since the eggs become more brittle the longer they remain in alcohols above 80 per cent, one should not prolong their residence in the highest strengths of alcohol—90, 95 and 100 per cent. Only at the sea-side do I use absolute alcohol; inland, even before clearing with toluene and xylene, I use only 95 per cent alcohol.

The 80 per cent alcohol is removed from the eggs and replaced by 90 per cent alcohol for 30 minutes. This is removed and replaced by 90 per cent alcohol, again for 30 minutes. The 90 per cent alcohol is removed and replaced by 90 per cent alcohol in the same way, except that the two changes are 15 minutes each. The second change of the 95 per cent alcohol is now replaced by two changes of absolute alcohol, each acting 15 minutes. The absolute alcohol is removed as thoroughly and as rapidly as possible and replaced by an oil used for clearing, xylene or toluene, for example, two changes, acting 15 minutes each.

LITERATURE

JUST, E. E. 1930. The amount of osmic acid in fixing solutions necessary to blacken fat. **Science, Vol. 71.**

———. 1933. A cytological study of effects of ultra-violet light on the egg of *Nereis limbata*. **Zeitschr. Zellf. mikr. Anat., Bd. 17.**

MATHEWS, A. P. 1901. The changes in structure of the pancreas cell. **Journ. Morph., 15. suppl.**

VI

METHODS OF CLEARING AND IMBEDDING

The present section deals with the simplest methods of clearing and imbedding eggs. The reader may find these directions too detailed and tiresome. It is nevertheless necessary to follow them because in the clearing and the imbedding of minute marine eggs, especially at the sea-side, a small mistake will undo the long process that has gone before. The care exercised in the preparation of sections is repaid by the ease and pleasure which one enjoys in studying perfect preparations.

Clearing

The choice of clearing agents is wide. One may use among others oil of bergamot, cedar-wood oil, chloroform, toluene or xylene. Bergamot is useful for it will clear after 90 per cent. alcohol. Some of my best preparations are of eggs removed from 95 per cent alcohol and cleared in cedar-wood oil. Both of these oils may be refractory at the sea-side even when one has the best grades. When used they should be followed by toluene or xylene before imbedding in paraffin. With chloroform one must work quickly to avoid the taking up of moisture. On the whole, the worker can depend upon toluene or xylene both for clearing and for infiltrating the eggs with paraffin. The objections often made against their use do not apply in the case of minute objects such as marine eggs, provided the eggs do not remain too long in them. One should however be at great pains to employ only the best grade of toluene or xylene which is acid-free. Kept in large bottles, small volumes of either tend to become acid in time.

In the bath of the first toluene or xylene the absolute alcohol is replaced by the oil. The small volume of alcohol remaining should mix thoroughly with the oil without any trace of murkiness due to the presence of water. The second bath insures

perfect clearing of the eggs. From this they are removed to melted paraffin for the imbedding.

Imbedding

From the second toluene or xylene the eggs are removed by a pipette thoroughly dried by bringing it into a flame and expelling the air from the bulb. Xylene or toluene is then sucked up into the pipette, care being exercised that the oil does not get into the rubber bulb. The drop of eggs is now pipetted into the receptacle to be used for imbedding. In carrying over the drop of eggs to the receptacle there will be some xylene or toluene present. This is no serious disadvantage, unless it takes up moisture from the atmosphere. To avoid excessive evaporation of the xylene or toluene brought over with the eggs, the receptacle may be covered with a thin glass bowl from which the water vapor has been driven off previously. No eggs should remain in the tube from which the drop has been transported, especially in those cases in which only a few eggs have been carried through. Also, no eggs should stick to the pipette used in making the transfer. The danger of loss of eggs in this wise may be lessened if the pipettes used are scrupulously clean and perfectly dry, and if in addition before being used for transferring the eggs they are kept in xylene absolutely free from any trace of water. The slightest trace of water will tend to make the eggs stick to glass.

For imbedding minute eggs workers employ various receptacles and devices. For example, some embryologists imbed the eggs in glass vials which they break after the paraffin has hardened—a miserable method. Still another method is to imbed the eggs in a dish which has a trough in the bottom into which the eggs settle. Other workers use gelatin capsules. Or they imbed the eggs in a packet of frog's epithelium. The disadvantage of these three latter methods appears when one cuts the paraffin blocks: the sections often do not ribbon properly. In addition, it is a nuisance to trace continuously all the slices of one egg from one row of sections to another especially where many eggs are present. As often is the case, the simplest method is the best. This is to use watch crystals with flat bottoms of about 4 centimeters diameter having a capacity of 10 cc. Or

one uses salt cellars selecting those with most wide-flaring sides and flat bottoms. The salt cellars are easier to handle but the watch crystals giving a more quickly solidifying block of greater evenness are preferable. The watch crystal should be thoroughly dried by holding it over a flame before the eggs are deposited in it. *Avoid over-heating the crystal!*

The crystal containing the eggs is placed on an electric hot-plate, the temperature of which is 60°C. Before the eggs become dried they should be flooded with paraffin melting at 60°C. In this they are gently shaken up to insure diffusion of what oil remains, then the dish is rotated to bring the eggs into a solid drop. Paraffin is then removed and replaced by fresh. The process of shaking the eggs, rotating the dish is repeated, paraffin is removed again; the process repeated. This threefold changing of the paraffin should not take more than 15 minutes, ample time for the infiltration of the eggs by the paraffin.

It is of the utmost importance to use the best grade of paraffin for imbedding eggs. Secure therefore from a reliable dealer a transparent paraffin guaranteed to have been filtered. That which comes in closely sealed tins is most practical; if it comes wrapped in paper, remove it carefully to avoid its taking up moisture, melt and filter it to insure its freedom from dirt. Pour the melted paraffin in a tin with a tightly fitting cover.

The melting point of the paraffin used is determined by the temperature at which it is to be cut, the quality of the object to be imbedded and the thinness of the sections to be made. In general, one takes a paraffin of high melting point (a) for cutting sections in the summer or in a warm room; (b) for brittle or hard objects and (c) for cutting thin sections. Since the eggs after fixation with the modified Meves are somewhat brittle and hard and are best cut into sections from 3 to 6 microns either at the temperature of the summer or in a warm room in winter, I recommend the use of a paraffin melting at 60°C.

Also for less brittle and hard eggs, as those fixed with Bouin or Carnoy or other fixatives, paraffin of 60°C. melting point is not too hard: for such the room temperature at the time of cutting is easily adjusted. In addition, it is true that a paraffin of 60°C. melting point is much easier and quicker to manipulate than one of a lower melting point. In my experience, paraffin

melting at 60°C. in no wise impairs the quality of the fixed egg. The notion that this temperature injures the already dead egg is in my judgment wholly illusory. So too, the belief that celloidin is superior to paraffin as an imbedding medium.

I have made sections of two lots of eggs in the same stage of development, one lot imbedded in celloidin and the other in paraffin. Careful study revealed that the eggs imbedded in celloidin were in no wise superior to those imbedded in paraffin. The cumbersome, tedious method of celloidin-imbedding certainly has advantages over paraffin in the case of some objects; these do not obtain in the case of the minute eggs under discussion. Indeed, I find that even for some objects refractory to paraffin imbedding, one need not have recourse to celloidin: Johnstone's rubber-asphalt-paraffin mixture serves exellently. Also, the use of chloroform or of cedar-wood oil for clearing—the second followed by toluene or xylene—will lessen some of the difficulties encountered in imbedding in paraffin and so obviate recourse to celloidin.

The trick, if it may be called such, in using paraffin for imbedding eggs at the sea-side, is to employ, I repeat, glassware of absolute cleanliness and to avoid moisture and over-dryness (from the xylene or toluene) of the eggs when subjected to paraffin infiltration. Manipulate with quickness and deftness in order to prevent excessive exposure which again increases the chances of water being introduced into the paraffin mass.

Not one single air bubble should be allowed to remain in the paraffin mass; to this end, prick with a hot needle any such bubbles. Also, the paraffin must not be allowed to crystallize. Either air bubble or crystals in the paraffin block render good sectioning difficult. The cooled paraffin block should be perfectly transparent.

This method of imbedding animal eggs of minute size is the simplest that I know. If one prefers a more complicated method, one has the choice of several. But I think that the results will not be superior. Indeed, I have tried several methods, described in the literature, and have used the best forms of paraffin baths available. I have never had results any better than those obtained with an ordinary copper plate heated with an alcohol lamp. Used with care this is as good as the electric hot-plate

mentioned above. The chief difficulty to be overcome is that due to the presence of moisture. For this reason I am very careful with the absolute alcohol that I use. I keep it in glass stoppered bottles of not more than 250 cc. capacity sealed with wax or paraffin. 95 per cent and absolute alcohol take up moisture very quickly at the sea-side. The xylene or toluene is not so hydroscopic. The great danger comes not from the xylene itself taking up the moisture but from the fact that the absolute alcohol contains moisture and this is carried over when the eggs are transferred from the absolute alcohol to the xylene or toluene.

Preparation of the Paraffin Block

A moment after the last paraffin has been added to the eggs, the dish is carefully removed from the hot-plate and gently lowered into a finger bowl containing 80 per cent alcohol and surrounded by one part cracked ice and two parts water in a dish of about 2 liters capacity. At first only the bottom of the crystal containing the eggs is immersed in the cooled alcohol. Slowly more of the crystal is lowered until a solidifying film appears on the upper surface of the paraffin. Then the crystal is carefully tilted to allow alcohol to run over the hardening surface without breaking the newly formed film or even making a ripple on it. Now the crystal is cautiously released and fully submerged. The alcohol is next covered with a glass plate. In a few minutes the block of paraffin becomes dislocated and floats. If it does not separate from the crystal, pressure of the finger on the rim of the block may dislodge it. Should it still not come away, cut around the rim of the paraffin with the point of a knife and gently press on the edge with the fingernail. The failure now to dislodge the paraffin block means improper infiltration of the paraffin, presence of xylene or of alcohol: the paraffin may come away leaving all or some of the eggs adhering to the glass.

If one immerses the congealing paraffin mass in a too cold medium, as ice and water or cold running water, too rapid cooling of the mass obtains with the result that the paraffin block cracks. The presence of these fissures is an obstacle to good sectioning.

With the cooled alcohol the paraffin never cracks. But do not allow the alcohol to become too cold.

The paraffin block is now labelled by scratching with a fine needle on each flat surface near the mass of eggs. It is then trimmed with a sharp knife with that surface of the block uppermost nearest which the eggs lie. Along one side the paraffin is cut away to a tangent one millimeter away from the black patch of eggs. From the edge opposite only enough paraffin is removed to make this parallel to the first. On the other sides the block is so trimmed that it assumes wedge-shape, the upper borders of the inclined planes coming not closer than one millimeter to the eggs at that point where they lie closest to the surface first cut. Thus one prepares a wedge-shaped block in which the eggs show up nearer one parallel edge than the other; these edges we may designate cutting edge and affixing edge respectively.

The face of the block opposite that nearer which the eggs lie may now be trimmed with a very sharp knife. The trimming, accomplished by paring away less than a millimeter of the paraffin at a time, extends only about one third the distance from the cutting edge to the affixing edge in order that this latter may be solidly stuck to the mounting disc of the microtome. The paraffin is pared away evenly so that the faces of the block are as nearly parallel as possible, the block having a thickness between the two faces of about two to two and one half millimeters. Any further trimming is accomplished after the block is affixed.

The block is now fixed to the disc. This is accomplished by dipping the surface of the disc into melting paraffin and gently pressing the paraffin block on to this surface as the melted paraffin congeals. Once the joint is made, it is further strengthened by surrounding it with melted paraffin. The mass of paraffin should now be one fused block. The disc is then put upright in a cool place—but never in cold water for this breaks the joint! —and allowed slowly to solidify. If the mount lies on the side during this process of cooling the paraffin block may bend; for this reason I prefer to use discs that can stand upright instead of those the ends of whose shanks are convex.

Cutting Serial Sections

Much may be said in favor of the so-called precision (sliding) microtome, but where many serial sections are demanded as in the case of several thousand minute eggs, an automatic rotary microtome may be employed to advantage. First of all, one saves much time by using such. Also, it gives better ribbons and minimizes the chance of losing sections. And if the instrument is a high-grade one, properly cared for, it will cut sections of uniform thickness, without skipping. I recommend the Spencer Automatic Rotary Microtome. With three different microtomes of this type I have cut perfect serial sections of three or four microns without any fear that sections jam, fail to ribbon or come off double the thickness for which the instrument is set. Doubtless many another high-grade automatic rotary is capable of equal performance.

The disc is mounted on to the microtome *with that flat surface of the paraffin block nearer which is the patch of eggs downwards.* In this wise, the knife cuts first through the thin layer of paraffin underlying the eggs, then through the eggs and last through the thicker paraffin layer above the eggs. If the block is mounted the other way around, the sections do not ribbon properly. Once the knife is in place, the block is adjusted by means of the screws of the disc-holder so that the lower edge of the block is parallel to the edge of the knife. The block is now most firmly clamped in place.

For serial sections of eggs, each of which is a composite of oil, yolk and granules of different physico-chemical make-up, in a more fluid menstruum, so fixed that not only the nucleus with its components and the cytoplasm are preserved but also the various cytoplasmic inclusions, it is absolutely mandatory to use the best microtome knife available. Such substitutes as safety razor blades are interdicted; they merely tear through the eggs, dislodging the oil-drops and yolk-spheres now of a stone hardness due to fixation, dehydration and clearing. Indeed, such thin blades often dislodge chromosomes and spermatozoa attached to the eggs. These imitation microtome knives do very well for objects so fixed that they are vacuolated masses, the oil, yolk and granules having been destroyed; but

for a well-fixed egg which is a mixture of components of varying degrees of consistency one must have a real microtome knife and not a makeshift, especially for thin sections. Secure therefore a heavy knife of the very best steel; both money and time are thus eventually saved. I use knives of not less than 12 cm. in length, 6 cm. in height; frequently for very brittle and hard objects those of 26 by 8 cm.

For cutting, the knife is most firmly clamped. The knife-carriage is then brought to within two millimeters of the forward edge of the paraffin block. With even, gentle strokes the wheel is turned with the cam set at 15 to 20 microns until the first section is cut. The cam is now reset for the thickness of the sections desired and cutting begun anew. Since these first slices contain no sections of eggs, needed adjustments can be made—of the alignment of the block and the tilt of the knife.

For cutting serial sections the knife should be slightly tilted toward the paraffin block. It is difficult to set a definite rule concerning the degree of tilt: so much depends upon the size, weight and form of the knife as well as upon the hardness of the paraffin and the heterogeneity of the object. One must therefore by experience *avoid excessive tilt.* Set the knife at that angle which gives a smooth ribbon in which the sections of eggs are perfectly intact. And as always be sure that the edge of the knife is as sharp as possible. Despite the utmost care one often includes with marine eggs, especially those of echinoderms and of tubiculous worms, particles of grit or sand which in cutting nick the knife. For this reason, one must change the knife to a new position the moment that a rift appears in the paraffin ribbon; if this is not due to an air bubble or drop of water it is probably due to some hard foreign particle. In the long run it is safest and most economical to have experts resharpen the knives.

The sections should be cut without the loss of a single section in one continuous ribbon whose length is sufficient for mounting on one slide. The ribbon is removed with camel's hair brushes on to smooth hard paper and cut into two, three or four strips, depending upon the width of the ribbon, each of which, allowing for the stretching of the paraffin, will make a row of sections slightly shorter than the length of the cover slip

to be used. On one slide should be placed as many sections as possible.

Mounting the Sections

The best microscope slides available should be used. Avoid if possible the use of those of a greenish tint. Procure therefore clear white slides with ground edges, free from defects. It is a good plan to select from the boxes of slides when first opened those of uniform thickness discarding the thicker ones and those that are too thin. Those selected are washed singly with clean hands in warm water and Castile soap, thoroughly rinsed and placed upright in covered jars of 95 per cent alcohol. If however the slides remain murky, they are placed one by one in jars of fuming nitric acid for about 24 hours. Next they are washed in running tap-water, care being exercised not to scratch them. They are then stored in 95 per cent alcohol until needed.

The slide to be used is removed from the alcohol which should run off without beading. It is then dried with clean bird's-eye cloth free from lint. Next a drop of Mayer's albumen fixative is conveyed to it by means of a glass rod. The drop of fixative is rubbed evenly over the slide by the index finger previously washed in soap and water and cleansed in ether. For sections that have been fixed in solutions containing chromic acid one uses more, and for those containing picric acid or formol, less, of the albumen fixative. Experience alone teaches the amount to use; too little means loss of sections, too much clouds the sections with stained masses of albumen.

The strips of paraffin are now placed consecutively on the slide in as nearly parallel rows as possible, the first cut end of each strip being about 5 millimeters away from the left end of the slide. Three or four drops of distilled water are run under the sections and the strips brought more closely parallel. The slide is now gently warmed by holding it above the electric hot-plate or over the feeble flame of an alcohol lamp. *But the paraffin must not melt!* In this wise the ribbons stretch and become easy to arrange in perfectly straight parallel rows. The water is drained from the slide; the slide is gently warmed again to insure equal adhesion of the strips throughout their extent; it is labelled on the right by scratching, with a splinter

or carborundum or with a diamond, the legend or number of identification. It is then placed section side uppermost, in a dust-proof slide-box which stands upright. It should remain thus for 24 hours when it is ready for the manipulations preparatory to staining.

Preparation of Slides for Staining

It is convenient to carry through for staining at least ten slides at a time but not more than twenty.

First the paraffin is removed. This is accomplished by immersing the slides in turpentine, gasoline, xylene, or toluene or other paraffin solvent. For most purposes xylene or toluene is to be preferred. One gives the slides two consecutive baths in xylene of ten minutes each. What follows depends upon the stain to be used.

Staining solutions employed are either alcoholic or aqueous. The simple rule to be followed therefore is to carry the slides from the second bath of xylene to two successive baths of absolute alcohol, 10 minutes each, and from these to a bath of 95 per cent alcohol. Here one stops if the stain is dissolved in 95 per cent alcohol. If not, the slides are carried through successively weaker baths of alcohol—80, 75, 50, etc.—to that strength of alcohol in which the stain is dissolved. If the stain is an aqueous one, the slides are carried from 50 per cent alcohol to 35 per cent and finally to water. In the staining methods given beyond, the stains are dissolved in 50 per cent alcohol and in water. Hence the manipulations preparatory to staining call for the treatment of the slides successively, after residence in the second absolute alcohol, in alcohol of 95, 80, 70, and 50 per cent each for 10 minutes for staining in the alcoholic solution and for a subsequent treatment in 35 per cent alcohol and in water for the same duration of time for staining in the aqueous solution.

VII

METHODS OF STAINING

The staining of eggs may be discussed from two points of view; staining of the living egg and staining of the fixed.

Intra-Vitam Staining

Living cells are often stained with dyes which do not impair their viability. Among such stains are methylene blue, neutral red, janus green, nile blue sulfate, etc. The precaution to be taken in using such vital dyes is to avoid concentrations that are toxic. The stains are therefore made up in sea-water in such way as to give the barest perceptible tinge of color. Wherever possible dyes employed should be of the same manufacture. Even so, the proper concentration has to be worked out for each case, because these dyes vary greatly. The stained eggs should be as perfectly viable as the normal unstained. No set rule can be given as to the exact dilution of dye. One should make up a series of dilutions, taking the most dilute that stains the cell. If this be toxic, as shown by failure or any abnormality of development, this particular make of dye should not be used; another should be tried.

Staining of Fixed Eggs

Fixed eggs may be stained *in toto* or after having been sectioned.

In toto staining

In toto staining is often very useful. It may be the sole method for the study of the fixed egg or may be employed as an aid for the study of stained sections. In the former case the eggs are best fixed in solutions which do not contain osmic acid, since this blackens the egg to such an extent that details of structure are not easily visible. One should rather use fixatives

made up with mercury bichloride, as Lang or Gilson, or those containing picric acid, as for example, Bouin's. Osmic acid alone or in combination with chromic acid or potassium bichromate is excellent for the study of the egg's contour and for changes at the surface. As an aid for the study of the sectioned egg, *in toto* preparations are helpful since they serve as a rapid means for determining stages which one desires to study in greater detail. Obviously, *in toto* preparations give only gross pictures. The practice by some so-called experts in cytology of using *in toto* preparations for the investigation of such fine cellular structures as asters and centrospheres is to be condemned. And certainly, where the whole eggs are subjected to pressure—itself an experimental method—in order that they may be studied under apochromatic oil-immersion lenses, interpretation is even less valuable.

The stains employed for *in toto* preparations are usually borax carmine and Ehrlich's or Delafield's haematoxylin. These bring out grosser structures very well. For the study of finer cell structures, however, other dyes are to be preferred.

Staining of the sectioned egg

Stains employed for the sectioned egg may be classed as nuclear stains, plasma stains and stains for mitochondria and Golgi apparatus. What was said above with respect to fixatives may be repeated here: no single stain serves perfectly to answer all purposes. Nevertheless, Heidenhain's iron haematoxylin stain is the nearest approach to an all-purpose stain, provided the fixation is proper.

(*Nuclear stains*)

Of nuclear stains, haematoxylin is most commonly employed. Safranin is excellent especially for phases of mitosis. Gentian violet is comparable to safranin. The Flemming triple stain, although often difficult to manage, gives results that repay the worker for the time consumed in learning its use. The same is true of Ehrlich-Biondi. Bensley's various combinations of dyes and Pianese's staining methods, especially when followed by his fixatives, have given me beautiful preparations. Here I emphasize the methods which have proved most useful to me. These

are: Heidenhain's iron-alum haematoxylin and safranin (or gentian violet).

(a) Heidenhain's Iron Haematoxylin.—Heidenhain's iron haematoxylin is a 0.5 per cent aqueous solution of the dye. I find that the solution may be made with tap water or distilled water. Although many workers prefer to use the solution only after it has been standing for some days, I get best results with a freshly prepared solution.

Before coming into the stain, the sections, previously washed in water, are kept in 4 per cent aqueous solution of iron alum for 15 to 60 minutes, depending upon their thickness. The sections are now carefully rinsed in water to remove the superfluous iron-alum. They are then brought into tubes of the staining solution for 1 to 16 hours, depending upon the depth of color one desires to obtain; shorter baths in the iron alum and in the dye stain the chromosomes blue, longer ones, black. As will be noted beyond, the shorter baths stain plasma and cytoplasmic inclusions after fixatives containing osmic acid and chromic acid as that of Meves.

From the staining solution the sections are brought into water and thoroughly washed. This is best accomplished in a large volume of water where the slide is kept until no more color is discharged. The slide is now brought into a 2 per cent solution of ferric alum and then placed under the low power of the microscope illuminated with daylight. The ferric alum brings about discharge of color. This should be carefully controlled by dipping the slide into water which arrests destaining. (Caution: Filter both ferric alum solutions before using them!)

At the point of the desired differentiation, the slide is removed to a tube which is placed under a gentle stream of tap water for one hour. After this, the slide is run up through successive grades of alcohol: 35, 50, 70, 80, 90, 95, 95, 100, 100, percent, each for 5 to 10 minutes. The sections are cleared by placing them in two changes of xylene or toluene for 5 minutes each. They are then mounted in a thin solution of neutral xylene balsam.

1. *Author's modification of Heidenhain's iron haematoxylin.*—I frequently take, instead of a 0.5 aqueous solution, as used in the Heidenhain method, a 0.25 or a 0.125 aqueous solution.

I use this freshly prepared in either tap or distilled water. In my hands, this modification is superior to the orthodox Heidenhain method. Mordant and destaining are unchanged. (Just, 1933.)

(b) *Safranin.*—Safranin gives beautiful preparations especially of chromosomes in stages of mitosis. However, it often proves a very refractory stain. When successful its brilliancy renders the study of mitotic stages a real pleasure. I have used all of the various recipes given for safranin, but I have never obtained with any one of them preparations as beautiful as those furnished by the method which I myself have developed for this dye. Usually one is advised to secure safranin of a certain serial number, as for example, Safranin o of Grübler. This is then used in distilled water saturated with aniline oil or saturated in alcohol or made up of equal parts alcoholic and aqueous solutions. With either of these methods the same safranin used does not give constant results. In such cases of failure one is often advised that now the aniline oil is at fault. The failures can be overcome with perfectly constant results by using my method which follows:

1. *Author's method for safranin (or gentian violet).*—The cells, not only eggs, but others from Protozoa to human tissue cells, are fixed in modified Meves solution, as given above, or in Altmann. After washing in water and running up to 80 per cent. alcohol, the cells are brought into double distilled aniline oil. The aniline oil is changed five times. It is then replaced with xylene or toluene and infiltration with paraffin follows. The paraffin sections are treated as outlined above and the slides thus brought to 50 per cent. alcohol. From this they are removed to the safranin stain made up as follows: Equal parts of safranin saturated in 95 per cent. alcohol and of safranin saturated in distilled water, *without aniline oil.* The farther treatment and differentiation of the sections are with acid alcohol and clove oil, as given for the orthodox method for safranin staining.

For gentian violet proceed in the same way. Sections of cells cleared in aniline oil, as given above, take Flemming's triple stain very beautifully.

Plasma Stains.—The most commonly employed stains for the cell plasma are eosin and Orange G. These are both easy

to handle. A very beautiful plasma stain is given by Bordeaux red. For details concerning these and other plasma stains, the worker may consult standard text books. I find that Heidenhain's iron-alum haematoxylin gives clear pictures of the plasma. One can obtain dark blue chromosomes on a beautifully transparent, brilliant robin's egg blue plasma background superior to that given by any plasma stain. One needs only to time the duration of the baths in the mordant (4 per cent ferric alum) and in the stain. The differentiation should not be allowed to proceed to the point at which the plasma appears yellow or yellow-white but should be terminated when the plasma is grey. The subsequent washing, dehydration and clearing will give the desired bluish tint to the plasma. If it does not, the tap water should be made slightly alkaline. These results are best obtained after fixation with the modified Meves. However, some of the most brilliant plasma staining with Heidenhain's haematoxylin that I have obtained is that of cells fixed in Bouin and cleared from 80 per cent. alcohol in aniline oil—a method which years ago I recommended to other workers. For example, Dr. Ezra Allen used it at my suggestion with great success on mammalian cells. Although it is sometimes valuable to use two stains, one for nucleus and one for plasma, for the experimental embryologist whose primary aim in using sectioned eggs is to check observations on the living by study of the fixed, I believe that the single staining with Heidenhain's haematoxylin suffices.

Stains for mitochondria and Golgi apparatus.—The staining of cell inclusions, as mitochondria and Golgi apparatus, is generally regarded as extremely difficult. I should say that of all structures in cells these prove to be the most exasperating because the results are so varied. Failures are usually attributed, as in the case with safranin staining, to the quality of dyes employed. Undoubtedly, this is a factor. Nevertheless, it is true that two workers using the same dye and following exactly the same procedure, often obtain different results. This is especially the case with the Benda method for staining mitochondria and to a less degree with Altmann's simpler method. After the Benda method, however, the worker can depend upon perfectly constant results by using the method given above for successful safranin staining, namely, to clear the

eggs from 80 per cent. alcohol in aniline oil. Staining with the Fuchsin S and the counter-staining are carried out as given originally by Altmann or by Meves except that aniline oil is omitted from the water in which the Fuchsin S is dissolved, if the object has been cleared in aniline oil.

Heidenhain's iron haematoxylin is in my experience as good as, if not superior to, these special techniques for mitochondria or for Golgi apparatus, if it is used after the modified Meves or the Meves without glacial acetic acid. It is also successful after Altmann's fixation. In all these cases the cells may also be cleared in aniline oil from 80 per cent. alcohol. It is sometimes best, however, to replace the aniline oil not with xylene or toluene but with chloroform.

Stains for Spermatozoa

Depending upon the fixative employed Heidenhain's iron haematoxylin will bring out perfectly all of the structures in motile spermatozoa. In some cases, however, it is advantageous to employ stains in combination, as Flemming's triple stain. After modified Meves, Altmann or Regaud, iron haematoxylin brings out perforatorium, head, middle-piece and tail of the spermatozoon very sharply. I have kept spermatozoa of marine animals in Regaud for more than 20 years, in a solid cake. By dissolving a bit of this dry material in water on a slide and staining this, I have obtained a preparation in no wise inferior to that of the freshly fixed and stained spermatozoa. The great resistance of killed spermatozoa to change is well-known; spermatozoa burnt to a char maintain their shape.

Literature

Just, E. E. 1933. A cytological study of effects of ultra-violet light on the egg of *Nereis limbata*. **Zeitschr. Zellf. mikr, Anat., Bd. 17.**

APPENDIX

I

DIFFERENCES IN EGGS WORTHY OF NOTE

For experimental work on animal eggs it is well to bear in mind that the fertilization process varies depending upon the stage of maturation in which fertilization normally occurs. Eggs thus fall into four classes:

Class I, fertilizable in the stage of the intact germinal vesicle. Examples: Eggs of *Nereis*, *Thalassema*, *Myzostoma*, and *Mactra*.

Class II, fertilizable in the stage of first maturation. Examples: Eggs of *Chaetopterus*, *Mytilus*, *Cumingia*, *Ciona* and *Molgula*. Eggs of the genus *Asterias* belong here also.

Class III, fertilizable in the stage of second maturation. Example: Egg of *Amphioxus*.

Class IV, fertilizable after complete maturation. Examples: Eggs of echinids.

Too frequently experimentalists work on eggs of one fertilization class and incorrectly draw conclusions concerning eggs of other classes. Morphologically as well as physiologically, the process of fertilization is different for each class. Animal eggs even of the same fertilization class differ in other respects. These differences also should be kept in mind.

Thus eggs differ with respect to the presence or absence of jelly, the distance at which the membranes separate after insemination, changes in shape of the egg after membrane-separation, etc. Also, the egg may possess a micropyle through which the sperm enters; lacking this, sperm entry may be at a fixed point or not. A fertilization cone of larger or smaller size which persists for a longer or shorter time, may or may not form.

An egg at the moment of normal fertilization may or may not possess an aster or asters in association with its nucleus in

the resting stage or in a phase of mitosis. The sperm after entry may or may not be accompanied with astral configurations. When present the aster may or may not contain a central granule, the centriole. (Centrioles may be seen in some living eggs, as Breslau has shown.)

(1) Eggs which are enclosed in jelly before fertilization: Echinids, starfish, *Cumingia*, etc.

Eggs which extrude jelly after fertilization: The genera *Nereis* and *Platynereis*.

(2) Eggs with most pronounced membrane-separation: Echinids, *Asterias*, *Nereis;* with least: *Chaetopterus*, *Cumingia*.

(3) Egg with most pronounced changes in shape after fertilization: *Nereis limbata*. To a less degree: Echinids.

(4) Eggs with micropyles: *Loligo*, teleosts. (The funnel in the jelly of echinid eggs is *not* a micropyle!)

Eggs into which spermatozoa enter at a fixed point: *Ciona*, *Amphioxus*, and usually *Chaetopterus*.

(5) Eggs with most persistent fertilization cones in the order named: *Nereis*, *Echinarachnius*, *Arbacia*.

(6) Eggs whose nuclei show no astral formation at the time of fertilization: Those fertilizable in the stage of the intact germinal vesicle; *Ciona*, *Molgula;* and echinids. In *Ciona* and *Molgula*, the maturation spindles present are anastral; in the echinids, the resting nucleus shows normally no radiations until apposition of the sperm nucleus.

(7) Eggs in which the sperm nucleus shows no asters: Trematodes.

(8) Eggs in which the sperm nucleus is accompanied by an aster without centriole: Echinids. With a centriole: *Nereis*, *Chaetopterus*.

II

SUMMARY OF MEANS ELICITING EXPERIMENTAL PARTHENOGENESIS IN VARIOUS EGGS

On the basis of the classification of eggs according to the stage in maturation when the fertilization moment occurs (see I of Appendix), the following summary of single means for inducing experimental parthenogenesis may be made (the Roman numbers indicate the fertilization-class):

Means:	Eggs:
Hypertonic sea-water	*Nereis* I (without cleavage)
	Chaetopterus II (without cleavage)
	Asterias II
	Echinoidea IV
	Mactra I
Heat	*Nereis* I
	Chaetopterus II
	Cumingia II
	Asterias II
Carbon dioxide	*Asterias* II
Butyric acid	*Asterias* II
Acids in sea-water	*Thalassema* I

Hypertonic sea-water is most generally effective, although in some eggs it does not induce cleavage. A named means effective on one species of egg of a given class may be ineffective on that of another member of this class. Thus it is apparent that the means can not be correlated with the stage in which the egg is fertilizable, which is the only stage when experimental parthenogenesis can be induced.

III

PROTOCOLS ON FIXATION AND STAINING

In order to facilitate the use of the directions on fixation, imbedding, and staining given in this book, I present them in summary and topical form as "protocols" for (1) staining with iron haematoxylin, (2) with safranin, and (3) for staining of mitochondria. For each an egg in metaphase of first cleavage is suggested as the object to be employed; any other small object, as insect testis, a bit of mammalian tissue, may however be substituted—in which case the making of an outline drawing, as recommended for the egg, is omitted.

Protocol I.

Staining with iron haematoxylin.

Example of object: Eggs of a nereid or of an echinid in metaphase of first cleavage.

1. With the aid of a camera lucida make as quickly and as carefully as possible an outline drawing of an egg showing its shape, extent of its spindle area and the disposition and size of the cytoplasmic components. (This is done to compare the fixed egg with the living.)

2. To a drop of egg suspension in a vial, add 2 cc. of the modified Meves. (Avoid use of cork stoppers!)

3. At 5 minute intervals during the next 40 minutes, gently turn the bottles upside down two or three times to insure even penetration of the fixative.

4. After 40 minutes, decant fixative carefully. Fill bottles with distilled water. (If distilled water is acid, use tap water.) As soon as the eggs settle, decant water and add water anew. Repeat this process, gently turning the bottles upside down after each renewal of water. In this wise wash eggs for an hour.

5. Run up eggs in alcohol: 35 per cent, 50 per cent, 70 per cent, and 80 per cent—one hour each.

6. Replace 80 per cent alcohol with two changes of 90 per cent alcohol, each change for 30 minutes.

7. Replace 90 per cent alcohol with 95 per cent alcohol—two changes, 15 minutes each.

8. Replace the 95 per cent alcohol with absolute alcohol—two changes, 15 minutes each.

9. Replace the absolute alcohol with xylene or toluene—two changes, 10 minutes each.

10. Imbed eggs.

11. Section eggs.

12. Mount sections.

13. Dry sections.

14. Place slides in each of following:

(a) xylene or toluene—5 minutes (to remove paraffin)
(b) xylene or toluene—5 minutes
(c) absolute alcohol—5 minutes
(d) absolute alcohol—5 minutes
(e) 95 per cent alcohol—5 minutes
(f) 95 per cent alcohol—5 minutes
(g) 90 per cent alcohol—10 minutes
(h) 80 per cent alcohol—10 minutes
(i) 70 per cent alcohol—10 minutes
(j) 50 per cent alcohol—10 minutes
(k) 35 per cent alcohol—10 minutes
(l) water—5 minutes
(m) water—5 minutes
(n) 4 per cent ferric alum—15 to 30 minutes

15. Rinse the slides very carefully in 1000 cc. of water.

16. Place slide in 0.25 per cent aqueous solution of haematoxylin for 3 to 5 hours or for 16 hours.

17. Wash the slide in 1000 cc. of water.

18. Place the slide in 2 per cent ferric alum; mount it under low power of the microscope, illuminated by daylight, and control the destaining. Arrest the destaining at intervals by bringing the slide into water. When destaining has reached the point desired, bring the slide into tap water.

19. Wash the slide in gently running tap water for 1 hour.

20. Run up the slide in alcohol as follows: 35, 50, 70, 80, 90, 95, 95, 100, 100 per cent, each for 5 minutes.

21. Place the slide in xylene or toluene, two changes, each for 5 minutes.

22. Mount the slide in thin neutral balsam.

23. Cover with a number 0 cover-slip, 24 by 50 mm. The cover-slip should be water-free by having been held over a flame for a few seconds.

24. Place slide with the section-side uppermost in a well-covered slide-box.

Protocol II.

Staining with safranin.

Example of object: Eggs of *Chaetopterus* in metaphase of first cleavage.

1. to 5. as in the foregoing.

6. Replace the 80 per cent. alcohol with 5 changes of aniline oil.

7. Replace the aniline oil with xylene or toluene.

8. Imbed eggs.

9. Section eggs.

10. Mount sections.

11. Dry sections.

12. Place slides successively in xylene or toluene and descending grades of alcohol to 50 per cent, as in Protocol I.

13. Remove the slide from 50 per cent alcohol to safranin made up by taking equal parts of a saturated aqueous solution and of a solution saturated in 95 per cent alcohol. Stain for 24 hours.

14. Rinse the slide in 50 per cent alcohol. Differentiate under low power of microscope in daylight in 50 per cent. alcohol slightly acidulated by the addition of 2 to 3 drops of a 1 per cent. nitric acid solution, added to 100 cc. of 50 per cent alcohol.

15. Transfer the slide rapidly but carefully to 70, 80, 90 per cent alcohol.

16. Mount slide under microscope and flood it with clove oil.

17. Place slide in xylene for 10 minutes with 3 changes.

18. Mount in neutral balsam.

Protocol III.

Staining for mitochondria.

Example of object: Eggs of *Chaetopterus* or of *Nereis* in metaphase of first cleavage.

Proceed as in Protocol II as far as 12.

13. Carry slides from 50 per cent to 35 per cent alcohol.

14. Wash slides in distilled water.

15. Flood the slide with a saturated aqueous solution of Fuchsin S. Warm the slide gently at 60°C. for 2 to 3 minutes.

16. Bring the slide into 95 per cent alcohol, saturated with picric acid. Under low power of the microscope in daylight, control the differentiation, removing the slide when all color has been discharged except in the mitochondria.

17. Transfer the slide to 100 per cent alcohol for ½ minute, then to toluene with two changes, each of 5 minutes.

18. Mount the slide in neutral balsam.